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Dermatological and Serological Aspects of Allergy

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Contact Dermatitis versus Atopic Dermatitis

By R. L. MAYER, Summit, N.J.

Introduction

When allergists and dermatologists gather together to discuss problems of common interest, they invariably return to the one subject on which they have arrived at an almost complete disagreement, namely, that of neurodermatitis or atopic dermatitis. I was tempted to take out a sword I had long laid away, when I was asked by our Secretary to open the discussions on the problem of Atopic Dermatitis *versus* Contact Dermatitis. For more than 30 years I have been interested in, and argued over, the experimental aspects of this subject. Slowly I have come to the conviction that the disagreement between allergists and dermatologists is perpetuated primarily by misconceptions and misinterpretations.

The very formulation of today's subject, namely atopic dermatitis *versus* contact dermatitis, seems to me not correctly stated. There is indeed no opposition between the two skin reactions, on the contrary, they are quite closely related and most dermatologists and allergists generally do agree, at least on one point, that contact dermatitis and atopic dermatitis are of allergic origin.

If one thus considers that contact and atopic dermatitis are allergies, then one should automatically consider them as pathogenically and immunologically identical, namely as expressions of specific sensitizations. Oddly enough, exceptions to this statement are still voiced. To cite only one recent publication, *Rostenberg* (1) declares himself vehemently opposed to *Cooke*'s suggestion that contact dermatitis and atopic dermatitis are immunologically the same.

Definitions

Such disagreements, like many other disputes in the field of allergy, are due to the unfortunate but perpetual confusion of definitions.

It has been indeed necessary, ever since I can remember, to open discussions on controversial subjects in allergy with definitions of the terms and problems involved.

There is relatively little ambiguity insofar as the term "*contact dermatitis*" is concerned. The allergists and dermatologists of English and German speaking countries use this term to designate a manifestation of a specific hypersensitivity of the epidermis mostly to external causes. The corresponding French term, the dermite artificielle, includes not only this allergic epidermal reaction, but also all the non-allergic epidermal inflammations caused by mechanical, physical or chemical action of substances with primary toxicity. I want to make it clear that I shall restrict my discussions to contact dermatitis as an expression of allergy.

The problem becomes very confused in respect to the term "eczema", at least for those dermatologists and allergists who still believe that contact type of dermatitis and eczema are different entities. *Kreibich* (2) has described the history of the term eczema and its many fluctuating definitions in a masterly monograph in Jadassohn's Handbuch. Yet today the camps are divided into the unitarians, for whom dermatitis and eczema are identical, and the dualists, who consider dermatitis as being different from eczema. *Jadassohn*, *Bloch*, *Kreibich*, their pupils and many others are unitarians while *Darier* and many of his pupils have separated the eczematose, the true eczema, the eczema malady, from dermatitis.

Equal discordance is found in many circles over the definition of the term "neurodermatitis", and the many different closely related dermatoses such as prurigo simplex, prurigo Hebrae, prurigo Besnier, the diffused prurigo of Darier, and the generalized prurigo of Vidal, and perhaps also what the French have called the "lichenifications secondaires". This is understandable since neurodermatitis has been separated from contact dermatitis and eczema, not on experimental evidence, but exclusively on the grounds of clinical, histological and anamnestic observations.

In 1933, *Wise* and *Sulzberger* (3) suggested that neurodermatitis disseminata and all the different prurigos should be united into one entity, and proposed the term "atopic dermatitis". They have included infantile eczema into this new entity since it is a common observation that neurodermatitis and prurigos originate in a large percentage of cases with an infantile eczema.

The introduction of the term "atopic dermatitis" by *Wise* and *Sulzberger* consecrated a prediction of *Coca*. Even in his first publication *Coca* had pointed out that eczema and certain forms of drug and food idiosyncrasies would eventually be placed together with asthma and hay fever into the group of atopies. The conception of the new entity of atopic dermatitis, and the inclusion of infantile dermatitis within this new entity has been hailed by one group of dermatologists but strongly combatted by the others. Particularly the American allergists and dermatologists, accept it as a real progress, while many of the Europeans have never made much use of the term atopy.

The introduction of the "atopic dermatitis" as a new nosological entity profoundly altered the original meaning of the term atopy. The term atopy was restricted to allergies of the evanescent type, with the infantile eczema, an allergy of the epidermal delayed type was included. Was it an advance to include neurodermatitis in the so-called atopies? We do not believe it was. The world of atopy is *definitely shrinking* as the experimental work on allergic sensitizations expands.

When *Coca* (4) first added the term "atopy" to the already overflowing dictionary of the allergists, the cornerstones of his new edifice were (a) the belief that the so-called atopies are restricted to man, and (b) the conviction that they are subject to hereditary influences.

a) *Atopies Restricted to Man*

At that time the only form of experimental sensitization recognized by *Coca* was that of anaphylaxis, and it was then believed that this allergic manifestation in animals was different from the allergic diseases of man, such as asthma and urticaria. Although similar to the anaphylaxis of animals in many respects, allergic asthma and urticaria were obviously caused by allergens which were not capable of producing anaphylaxis in animals. *Coca* therefore stated that anaphylaxis was restricted to animals and that asthma and urticaria were strictly human diseases.

Today we know a great deal more abo[illegible]

[illegible] of animals to many agents

which have caused allergic diseases in man have been accomplished, and most investigators recognize that anaphylaxis in animals and allergic diseases of man are identical. Consequently, with advanced experimental methods, the first cornerstone of atopy crumbled the belief that atopies such as asthma or hay fever are restricted to man.

But then, as today, it was not possible to reproduce the typical picture of neurodermatitis or atopic dermatitis in animals. Is this a valid reason for concluding that neurodermatitis at least is an atopy restricted to man?

If we are still unable to reproduce neurodermatitis in laboratory animals at will, this is owing rather, I believe, to methodological shortcomings and to the rarity of investigators interested in experimental allergic dermatology. I only know of two experimental attempts to produce an evanescent type of dermatitis medicamentosa of skin sensitization. Together with *Jordan* (5), I produced a morbilliform exanthema in guinea-pigs with pig serum, and *Dienes* (6) accomplished the same effect with horse serum. However, these were not really reactions of the type of atopic dermatitis. But in dogs there is an obviously allergic disease which consists of a lichenoid eczema and urticaria often associated with asthma and hay fever, and which in many respects, at least clinically, resembles atopic dermatitis in human beings.

b) Heredity, Familial, Personal Factors

The second pillar of the edifice of atopy, and consequently that of atopic dermatitis, namely the great importance of heredity, is apparently more shockproof. The role of heredity in asthma, urticaria, hayfever, as well as in neuro-dermatitis, has always been emphasized by clinicians, and the dermatologists have always agreed that heredity and factors of personal disposition play a lesser role in contact dermatitis.

The frequency of personal and hereditary factors among patients suffering from atopic dermatitis and the rarity of these factors in contact dermatitis has been summed up in these phrases: In contact dermatitis, the causative agent is everything, the host is of little importance. In atopic dermatitis, on the other hand, the host is predominant and the allergen is of lesser significance.

But is it true that personal or familial factors do play such a modest role in *contact dermatitis?* If a group of people is exposed to

relatively light concentrations of certain skin sensitizing agents, then, as a rule, only a few individuals become sensitized, the particularly sensitive individuals, perhaps those with a fair complexion and a moist skin. Most others are more resistant to the sensitizing agents.

We encounter a similar individual predisposition or resistance to allergic sensitization among guinea-pigs. In many attempts to induce skin sensitizations with simple chemicals only a few particularly susceptible animals become sensitized, whereas others react only after repeated applications of the sensitizing agent. Just as certain human individuals and entire human races are relatively resistant to epidermal sensitization, so there are individual animals and even entire strains of guinea-pigs which strongly resist, while others readily succumb to experimental sensitizations of the skin to chemical substances. *Landsteiner* and *Chase* established and maintained a strain of guinea-pigs whose skin was much more readily sensitized to simple chemical compounds than those of the usual varieties.

While it is generally recognized that heredity and personal disposition factors are frequent in *atopic dermatitis* the various statistics differ considerably, depending upon the provenance of the clinical material being analyzed. This is very evident when we compare the results of two analyses of hereditary and familial factors in atopy based upon entirely different material. In the first, the cases were selected solely according to the clinical aspects and case histories, the sensitizing agent was mostly unknown. In the second analysis the only patients who were included were those whose atopic symptoms were caused by the same known sensitizing agent.

First Analysis

Baer (7) has analyzed 150 cases of atopic dermatitis in which the diagnosis was made from purely clinical data; in the majority of cases the causative agent was not established. 31 % of the patients had a personal history of allergy, rhinitis or asthma. 62 % had histories of allergic asthma, rhinitis, or atopic dermatitis among members of the family, and only 21 % had neither personal nor family history of other atopies.

Second Analysis

Since I have not found in the literature any recent statistical analysis of atopic patients, whose allergic manifestations were caused by the same agent, I shall refer to my own published results. It is well known that quite an important percentage of workers in fur factories concerned with the dyeing of furs with paraphenylenediamine, or with the handling of freshly dyed furs become sensitized to this dye, and develop contact dermatitis, asthma, neurodermatitis or a combination of these manifestations. I detected, in such factories, 221 persons suffering from dermatitis, asthma or a combination of asthma and dermatitis or neurodermatitis (8). 209 of these 221 had acquired these manifestations subsequent to employment in the dye factories, and only 12, or 5.7%, had a personal history of allergy.

Of the 221 persons, 101 suffered from asthma, 96 from dermatitis and 24 from a combination of asthma and dermatitis.

Personal history of allergy was present:

Among the 125 cases of an atopy	in 6 = 4 %
Among the 101 cases of asthma	in 3 = 3 %
Among the 96 cases of dermatitis	in 6 = 6.2%
Among the 24 cases of asthma plus dermatitis or neurodermatitis	in 3 = 13 %.

Unfortunately the number of combination cases of asthma and skin manifestation was quite small. We recognize the surprising fact that in this etiologically uniform group, contrary to the previous group chosen at random, the percentage of cases with personal history was very low, much lower than in all other statistics involving atopies with multiple etiologies. The percentage of positive histories of allergy in *Baer*'s statistics is indeed almost three times larger than that of this material (Table I). But we were equally surprised to find that the percentage of positive personal history of allergies was very low among the patients with pure asthma due to paraphenylenediamine sensitization.

Simultaneous Occurrence of Asthma and Contact Dermatitis Both Due to the Same Allergen

If the concept of atopic dermatitis is correct, that its particular clinical character is due to a sensitization of the skin in an atopic individual, then we would expect that an atopic individual would

react exclusively with atopic manifestations *to his specific allergen.* One would not encounter alternation or the simultaneous presence of asthma and contact dermatitis induced by a single agent in the same individual. But, interestingly enough, alternation of asthma and contact dermatitis produced by the same allergen does exist. I only need to cite a few instances. *Kreibich* (1c) has reported the case of a quinine allergy in which asthma was associated with an acute dermatitis. Tests were positive: the introduction of quinine into the nose caused a typical asthmatic attack and simultaneous patch tests with quinine were strongly positive. Another of his patients suffering from asthma and eczema due to horse dander responded to a dander patch test with an acute dermatitis. In my own observations of patients with asthma and skin manifestations, both induced by p-phenylenediamine, more than one half, or 60%, had an association of asthma with acute and chronic dermatitis; but in only 33% asthma was combined with atopic dermatitis. The coincidence of an atopic and a non-atopic sensitization to the same substance is not restricted to man. In experimental sensitization of guinea-pigs to a single chemical compound the evanescent and the contact type of sensitizations are often associated. We have observed such double sensitizations in guinea-pigs sensitized to paraphenylenediamine. *Landsteiner* and *Chase* (9) obtained sensitizations of the immediate reactivity in addition to skin sensitizations of the delayed type when they sensitized guinea-pigs by intracutaneous injection of picryl chloride, dinitro-chlorobenzene and other simple chemical compounds This combination of epidermal and anaphylactic reactivity thus appears to be much more frequent in both guinea-pigs and man than had previously been suspected.

Table 1
Personal and Family History

Disease	Family and Personal History			
	R.L.M		R. Baer	
	Cases	Per Cent	Cases	Per Cent
Contact Dermatitis	96	6 2		
Asthma	101	3		
Asthma plus Dermatitis	24	13		
Neurodermatitis			150	79

It is very important to recall this fact when we attempt to learn more about the etiology of atopic dermatitis. *Jadassohn* has already observed, and we have confirmed these observations in the clinic many times, that the skin of patients suffering from neuro-

dermatitis is much more resistant to multiple irritants than the skin of patients suffering from acute or chronic dermatitis, and even more resistant than normal patients. They show far fewer unspecific reactivities to heterogenous substances than the skin of other patients. Recently, *Sulzberger* (10) again emphasized that patch tests are generally negative in atopic dermatitis. This was not the case in our own experience with patients allergic to paraphenylenediamine. Here we found, indeed, quite a number of positive patch test reactions among atopic patients. In fact, 13 of 18 atopic patients suffering from a combination of asthma and eczema gave positive patch tests with paraphenylenediamine. We are tempted to conclude that specific patch tests are usually negative in clinical cases of atopic dermatitis, because the actual cause of this dermatitis was not found.

The Role of Exposure in Contact Dermatitis and Atopic Dermatitis

a) Quantitative Factors

A factor of considerable importance in the establishing of any sensitization is the intensity with which man and animals are exposed to the sensitizing agents.

In all naturally occurring sensitizations of the delayed type in man, the normal differences between easily and difficultly sensitized individuals vanish when the sensitizing power of the contactant is high or when exposure to the contactant increases. In certain factories where, for a long period of time, only a few individuals have occasionally acquired sensitizations through contact with a chemical substance, the number of sensitizations will increase suddenly when the noxious agent is used in higher concentrations, or when contact with this substance is intensified. We encounter the same reciprocal relationship between the resistance to sensitization and the intensity of sensitizing procedure during experimental sensitizations of guinea-pigs. As in human allergy individual factors may be eliminated by intensifying the exposure to the allergen. Indeed, when arsphenamine, primuline, paraphenylenediamine, picryl chloride, etc. are applied in sufficiently high concentrations or repeatedly injected, the skin of practically all guinea-pigs, including the more resistant ones, can be made sensitive.

With increasing exposure the number of "experimental" sensitizations of the atopic type increases likewise in humans. Nirvanol, for instance, is known to produce a morbilliform, urticaria-like erythema in 10 to 15% of patients when used for a short time *only* and in relatively low doses. But when the drug is administered in high doses and for a prolonged period of time, as for instance during the treatment of chorea, virtually 100% of the patients so treated become sensitized. Employees in those sections of fur factories in which dry, black-dyed furs are handled represent another example. Here only certain individuals acquire asthma, yet I have found that practically every worker suffered from asthma who worked in those areas of the manufacturing process in which concentrated fumes containing the highly volatile oxidation products of paraphenylenediamine were inhaled. Neurodermatitis is no exception and if, for reasons still unknown, certain people in these fur dyeing factories reacted primarily with a neurodermatitis, which subsequently may have alternated with asthma, then increased exposure to the noxious agent was followed by an increased number of cases of neurodermatitis

Thus, disposition, personal or hereditary factors do exist in contact dermatitis and atopic dermatitis as well. They become unimportant in both instances when the sensitizing impact increases.

Table 2

Dependence of the Type of Allergic Reaction upon the Route of Administration

I. Plant and Animal Products

Substance	Route of Administration		
	Contact	Inhalation	Injection or Ingestion
Atropine	D	A	D, A, U
Morphine	D	A	D, A, U
Quinine	D	A	D, A, U
Emetine	D, U	A	An.
Ipecacuanha		A	A
Essential Oils	D	A	D, A, U
Asparagus	D	A	D, A, U
Celery	D	A	
Silk	D, U	A	
Hair	D	A	
Feathers	D	A	
Fish	D	A	
Tuberculin	D	A	D, A, U

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Essential Oils	D	A	D, A, U
Asparagus	D	A	D, A, U
Celery	D	A	
Silk	D, U	A	
Hair	D	A	
Feathers	D	A	
Fish	D	A	
Tuberculin	D	A	D, A, U

Table 3
Dependence of the Type of Allergic Reaction upon the Route of Administration
2. Metal Salts

Substance	Route of Administration		
	Contact	Inhalation	Injection or Ingestion
Arsenic	D	A	
Arsphenamine	D	A	D, A, An.
Bismuth	D	A	
Mercury	D		D, A, U
Chromium Salts	D	A	
Ammonium-persulfate	D	A	
Potassium Iodide	D		An.

Table 4
Dependence of the Type of Allergic Reaction upon the Route of Administration
3. Organic Compounds

Substance	Route of Administration		
	Contact	Inhalation	Injection or Ingestion
Formaldehyde	D	A	D, U
Insecticides	D	A	
Phenobarbital	D		D, U
Sulfonamides	D	A	D, A, U
Novocaine	D		An
p-Phenylenediamine	D	A	D, A, U

b) Different Types of Exposure Determine Different Types of Allergy

During a previous symposium of this Collegium, I (11) have drawn attention to the fact that, whether a given antigen will produce the contact type or the atopic type of sensitization, depends largely upon the kind of exposure.

Allergists divide allergens into two main groups, namely, atopens and non-atopens, according to the allergies they usually produce. Most substances responsible for atopies including atopic dermatitis are inhalants or ingestants, and most non-atopens are identified with contactants. But there is no such thing as an intrinsic contactant; it is the pure hazard of natural exposure which makes a certain allergen either an atopen or a contactant. In effect, any hapten can produce, under propitious conditions, any type of allergy. The most powerful contactants, such as formaldehyde or Chloramine T, induce asthma when they are inhaled. Paraphenylenediamine, first noted for its strong asthma-producing properties when inhaled in fur dyeing factories, was only later recognized as

one of the most dreaded contact allergens when it comes in contact with the skin (*Mayer*, 8). Many other allergens behave in the same way. As seen from Tables 2, 3 and 4 it is the nature of the exposure which largely determines the type of allergy.

If the same substances can produce atopic allergies when inhaled, or contact dermatitis when in contact with the skin, then we may assume that the two types of exposure – inhalation and contact – elicit two different general reactions.

Importance of the Carrier for the Type of Sensitization

What is the nature of the differences between inhalation of an allergen and contact with the skin? First, let us consider the possibilities that we can exclude. We can exclude the possibility that the allergen produces atopic allergies upon inhalation, because it comes in closer contact with the sites of the atopic reaction when it enters the organism through the lungs or the intestinal tract than when it comes in contact with the skin. This is certainly not the case in atopic dermatitis. We can also dismiss the possibility that contact with the skin leads almost regularly to the delayed type of allergy since the allergen remains longer at the site of reaction as it enters the skin than when it enters through the lungs or the intestines.

The principal difference between sensitization by contact and sensitization by inhalation consists, it seems to me, in the composition of those reactive body constituents that the hapten first encounters, and from which it will choose the carrier in order to become a full antigen (*Mayer*, 12).

If a powerful hapten, such as formaldehyde, comes in [illegible]

[illegible] the more soluble procollagen and prokeratin. Only in smaller proportions and consequently in weak competition with keratin and collagen does the hapten encounter globulins and albumins.

Conversely, when formaldehyde is inhaled, ingested or injected subcutaneously, intramuscularly or intraperitoneally, it first comes in contact principally with albumins and globulins, much less with collagen and not at all with keratin.

According to *Lightfoot* and *Coolidge* (13), a gram of guinea-pig skin contains 20 mg. of collagen, whereas one gram of vital organs contain only 1.1 mg. Keratin is confined to the skin and is normally absent in organs.

Collagens and keratins on the one side, and albumins and globulins on the other, differ considerably in their chemical composition and particularly in their physical properties. Collagen and keratin are so-called fibrous proteins with their molecules arranged in fibrils and oriented in parallels which are stabilized by balancing forces between the adjacent peptide chains and micelles. It is this particular physical behavior and the molecular stability of collagen and keratin which maintain the form and structure of all tissues, in particular those of the protective epidermis.

In contrast, albumins and globulins are so-called globular proteins. Their molecules are not oriented as are those of the two previously mentioned proteins, but are folded and coiled without preferred direction; they are the principal components of non-structural, proteinic body constituents, especially of blood serum and other body fluids.

Formaldehyde, to keep to the chosen example of a potent sensitizer, readily combines with both types of protein, since both are rich in those chemical groups (though in different proportions), to which aldehydes become easily attached. Consequently, upon contact with the skin, formaldehyde will be attached principally to the proteins that prevail in skin, namely, keratin and collagen and their precursors, and the complete antigens formed will be mainly complexes of formaldehyde and keratin or prokeratin or formaldehyde and collagen or procollagen. Upon inhalation, ingestion, or injection, formaldehyde will combine primarily with the then prevalent globular proteins and consequently the complete formaldehyde antigen formed after inhalation, ingestion, or injection, will contain mainly albumins and/or globulins as carriers.

Thus the first obvious difference in the chemical composition of various complete antigens formed from all haptens is a difference in the chemical composition and physical structure of the *carrier* substance. This is quite understandable in the case of a substance like formaldehyde which possesses such high affinities for proteins, affinities that make formaldehyde one of the most potent antibacterial and antitoxic agent. But many other well-known sensitizers behave very similarly when in contact with body proteins.

Among the strong sensitizers are mineral and organic compounds as well. Chromates, persulfates and mercury salts, for instance, are examples of strong mineral sensitizers. Examples of strong allergens of organic nature are aromatic amines, diamines, aromatic nitro compounds and amino-phenols, phenylhydrazine and phenylhydroxylamine, quinones, quinone imines and quinone diimines, aldehydes, particularly those of lower molecular weight such as formaldehyde or acrolein, unsaturated compounds, as for instance certain plant products, detergents of various anionic and cationic character, medicaments such as sulfonamides, local anesthetics of the Novocaine type, arsphenamines, etc. This is indeed an impressive list. Included in this group are compounds that are not sensitizing agents in themselves; they are precursors and are transformed metabolically into directly active antigens, as for instance aromatic amines, amino phenols, many nitro compounds, sulfonamides and local anesthetics all of which belong to the large antigenic group of compounds of quinone structure.

In spite of the apparent heterogeneity of this group of inorganic and organic compounds, they all share one common chemical property of great importance: a strong affinity for proteins which is equal or similar in strength to that of formaldehyde. All have particularly strong avidities for fibrous proteins and many of them combine so firmly with the fibrous proteins of the skin that they form water-insoluble, heat and bacteria resistant complexes generally known as leather. In other words, all these compounds belong to the group of *tanning agents*. As a matter of fact, every known powerful and widely used tanning agent is contained in this group of strong sensitizers; the aldehydes, polyphenols, chromates, quinones, unsaturated compounds, and wetting agents. It is obvious that the tanning activity of many chemical substances of low molecular weight is proportional to their sensitizing properties (Table 5). The strongest sensitizers are at the same time the most powerful tanning agents, and derivatives of the strong tanning agents which have lost their tanning properties because of chemical hindrances have also lost their sensitizing power.

The chemical bonds by which haptens attach themselves to carriers to form complete antigens, are identical with those that transform skin into leather: namely, *cross-links* between tanning agents and keratin and collagen and their precursors; obviously the same chemical reactions are responsible for both the tanning and the

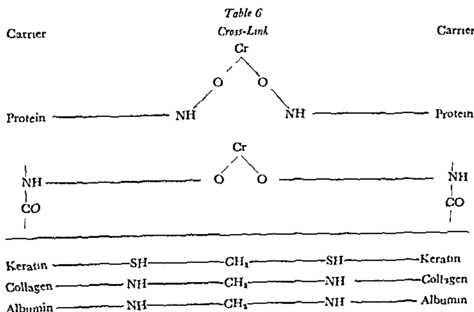
sensitizing properties of these compounds. In view of the parallelism between the chemical processes involved in sensitization and tanning we visualize the formation of a full antigen from a haptenic *contactant* and the skin proteins as follows:

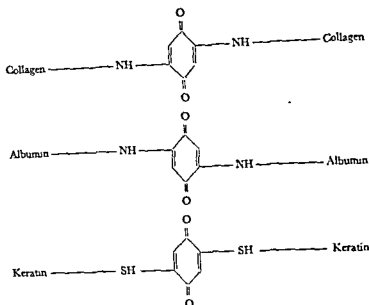
Table 5
Sensitizing and Tanning Activities

Group	Sensitizing Power	Tanning Power
1. *Aldehydes*		
Formaldehyde	++++	++++
Higher aldehydes	+	+
Aldehyde C_{18}	0	0
2. *Quinones*		
Ortho quinone	++++	++++
Quinone diimine	++++	++++
Di- and tetra-substituted quinones	0	0
3 Chromium salts	++++	++++
4 *Wetting Agents*		
Sulfonates	+++	+++
5 Unsaturated aliphatic compounds	+++	+++

Upon contact with and penetration into the skin, powerful sensitizing agents combine through cross-links with fibrous proteins and form insoluble (or at least difficultly soluble) complete antigens of the following, or multiples of the following constitutions (Table 6):

Table 6





These are the complete antigens of the contact-type of sensitization. We believe that in the case of atopic, anaphylactic sensitization haptens are transformed into full antigens by a very similar process, since it is known that all strong sensitizers, such as formaldehyde, quinones, chloramine T, persulfates, etc. undoubtedly combine, when inhaled or ingested preferentially with the globular proteins albumin and globulin to form complete antigens. In this way readily soluble antigenic globular complexes are formed which are associated with antibodies of the evanescent type; the insoluble or difficultly soluble fibroid complexes, on the other hand, formed by the combination of haptens with skin proteins, are associated with antibodies of the contact type of sensitization. Examples of both are shown in Table 6.

Chemistry of Full Antigens

We thus arrive at the following picture of the chemistry of full antigens. Although produced by the same chemical mechanism, namely the formation of cross-links, various complete antigens may differ considerably in the composition of the carrier moieties. Complete antigens formed on and in the skin contain predominantly fibrous proteins of the keratin or collagen families; whereas the

complete antigens formed in the lungs, intestines, organs, etc. contain globular proteins of the albumin and globulin families. The procollagen or prokeratin antigens are most likely highly viscous or even insoluble, since they contain cross-links with rigid macromolecules; but, the globular antigens remain soluble, because of their cross-links with non-structural macromolecules.

It follows therefore that the same hapten can form full antigens having different chemical compositions and different physical properties. Until now it has been the general consensus that the protein-carrier-moiety of a complete antigen does not play an active, specific part in the process of sensitization. The role of the carrier, it seems to me, is not merely a passive one. On the contrary, it is *much more likely that the cross-linking of the hapten with carriers* of various chemical composition and different physical properties sets the different patterns for the future form of the specific antibody.

According to one of the prevailing theories, antigens modify the enzymes of protein synthesis so that, instead of normal protein, antibody is formed. The antibody is, according to these theories, complementary in form and shape to the antigen. One may therefore assume that the antibodies produced by a complete antigen composed of a hapten plus globular protein carrier are different from those manufactured under the influence of complete antigens containing fibroid protein carriers. In the first case one may expect the formation of "globular", soluble and therefore humoral antibodies, and in the second instance, the formation of "fibroid", rigid, less soluble or insoluble, and therefore sessile antibodies. In other words, we may distinguish between complete antigens of the atopic type and antigens of the delayed type depending upon the chemical nature of their protein moiety as we distinguish antibodies of the humoral or the sessile type.

We have tested the correctness of this theory by a number of experiments in which we sensitized animals with antigens produced by combining picryl chloride with pro-collagen. When animals were sensitized by intra-peritoneal injections of this pro-collagen-hapten combination the number of sensitizations of the delayed type was equal to the number of similar sensitizations obtained after intradermal application of the hapten alone (*Mayer*, 14, 15, 16).

We have concluded from theoretical deductions and from the results of these experiments that the origin of an atopic or a non-

atopic allergy depends to a great extent upon the chemical nature of the protein to which a hapten is attached. A globular antigen, such as the native albumin or globulin antigen or a hapten combined with albumin or globulin produces an atopy, whereas a hapten combined with a fibroid carrier leads to sensitizations of the contact type.

The question naturally arises as to how the *atopic dermatitis* fits into this picture? I must admit that here again I can formulate only a hypothesis, since I have not been able so far to produce this type of allergy in animals. According to all available clinical and histochemical data, it appears quite probable that neurodermatitis is not a combination of the evanescent and the delayed type but a special type of sensitization.

In his clinic, *J. Jadassohn* always expressed the opinion that the reaction site in neurodermatitis is the reticulum of the cutis. The histology of dermatitis agrees with such a concept. Spongiosis and intra-epidermal vesicles, and other characteristics of the contact dermatitis are absent. Instead, in all typical cases of neurodermatitis we find strong, progressive hypertrophy of collagen within the cutis and later on mucilagenous degeneration as well as the altération cavitaire which in turn are lacking in contact dermatitis, urticaria and in angioneurotic edema.

Rappaport (17) has recently demonstrated characteristic changes in the histochemical behaviour of the mucoproteins of the connective tissue in atopic dermatitis involving a depolymerization of the ground substance and the basement membranes He considers these changes as evidence of an altered state in the glycoproteins and a loss of soluble material through the blood vessels.

Considering these specific changes of the connective tissue and the ground substance we need only a little analogy to consider that in the case of atopic dermatitis the haptens penetrating into the cutis, mostly from within, meet their carriers not among the keratins or albumins but among other constituents of the skin, perhaps the ground substances, mucopolysaccharides or lipopolysaccharides, etc. One may consider that in this manner still another complete antigen is formed within the skin besides the complete antigens containing globular or fibroid proteins and that this third type of complete antigen produces another specific type of antibodies which is responsible for the characteristic manifestations of atopic dermatitis.

From the chemical point of view, it is quite conceivable that all the well-known, highly sensitizing substances of low molecular weight and others may readily combine with mucopolysaccharides and similar constituents of the ground substances. They form very stable reaction products of which dioxymethin links are only one example.

I do not wish to fall deeper in the abyss of hypotheses and should like to return to my opening remarks and to the problem as it was originally formulated, namely, contact dermatitis *versus* atopic dermatitis.

We have discussed here two well-defined, clinical entities which have an immunological background in common. From an experimental point of view only contact dermatitis has been thoroughly explored and much knowledge has been accumulated in this field, by comparison of clinical data with the most interesting results of exact and successful scientific investigation. The field of atopic dermatitis is still wide open; here virtually all our knowledge derives only from clinical data, and no experimental attack has thus far been made. Before we are able to play one entity against the other, we must establish equal experimental bases for the atopic counterpart.

There are many other unsolved problems which I have omitted-willingly or otherwise. I beg your pardon for too much theorizing. But the fuel of progress is theories; many theories lead to dead ends but among the many, one may lead to the solution. I hope I have been able to present to you some of the many unsolved problems of the field of atopic dermatitis. I am certain that in the near future experimental research in atopic dermatitis will become an interesting and rewarding endeavor.

Summary

It is believed that much of the controversy regarding the relationship between contact dermatitis and atopic dermatitis is due to misunderstandings. Definitions are reviewed and the immunological aspects discussed. Both reactions are considered as allergic manifestations. Whether an allergenic agent produces contact or atopic dermatitis depends upon endogenous or exogenous factors. The same agent may produce contact dermatitis and atopic dermatitis as well, but it depends largely upon the type of exposure

whether a contact or an atopic type of sensitization is established. In the case of complex antigens, sensitization as such depends upon the haptenic moiety, the type of sensitization upon the carrier moiety to which the hapten is attached.

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titis» might have been replaced by: neurodermatitis, lichen Vidal, prurigo Besnier, endogenous eczema, spätexudatives Ekzematoid, etc. But I think that we can leave such a discussion behind us, for in the final analysis, names are not of essential importance, but rather, a true understanding of what one is talking about.

Allow me then, in order to facilitate my task, *to modify once again the title of this lecture.* I should like firstly to speak of atopic dermatitis, then of contact eczema.

The following quotation is from *Boyd:* "The term atopy, meaning «a strange disease», was proposed by *Coca* to denote certain clinical forms of human hypersensitiveness which are affected by a hereditary predisposition."

We often find in a single family different forms of atopy, e. g. disseminated neurodermatitis, infantile eczema (these are the terms which we use in our clinic), asthma, and hay fever. Every experienced dermatologist who has made case histories about neurodermatitis, with attention to the four above-cited conditions in the same patient, and among the other members of the family, will agree, I believe, that here one cannot properly speak of coincidence. Assuming of course, that the patient has at least a certain minimum of intelligence, that he has a family, naturally the larger the better, and that he has not fallen out with his relatives to the point of hardly knowing anything about them, there will be a high probability of learning from him that in his family there is some neurodermatitis or one of the other atopic manifestations. It is this fact, among others, which seems to establish the correctness of grouping together neurodermatitis, infantile eczema, asthma, and hay fever.

For the dermatologist who is specially interested in disseminated neurodermatitis it cannot be doubted that infantile eczema is intimately related to neurodermatitis, even without taking the family history into account. How often do patients with neurodermatitis say that in their earliest childhood they suffered from eczema? *Vowles, Warin* and *Aplay* investigated this in another way, and obtained very interesting figures. In 78 children who had infantile [illegible] edly, was [illegible] "asthma, [illegible] in 73% of the patients, as compared to 5–7% of compared groups". It seems to me that in the future it would be more correct to speak

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Contact Dermatitis versus Atopic Eczema

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I should like first of all to thank you very sincerely for your kind invitation to ask me to speak to you on: «Contact dermatitis *versus* atopic eczema», an invitation which has given me a great deal of pleasure, but at the same time, a certain amount of concern. There is little need to explain the reason for my satisfaction, however, so perhaps I should say a few words regarding the source of my concern. This is a difficult subject, one in which the prevailing opinions differ widely; and from the start, I had to put aside the possibility of entering upon all the subjects which might have appealed to me to pursue. This might have led me too far afield, and also, I must admit to you that due to lack of time, I was unable to consult the literature as much as I would have wished to. I must therefore apologize for referring more to my own observations than to those described in the literature And one further point: it is truly regrettable that ever since the unfortunate construction of the Tower of Babel, our native tongues have been so dissimilar, all the more so, since even dermatologists and allergists whose mother languages are the same, often speak different professional languages, while dermatologists on the one hand, and allergists on the other, frequently among themselves, speak their own various jargons. I assume that I shall not be required to attempt to prove what I have just said. However, if such a proof were called for, the subject of this lecture would be especially appropriate in this respect. We could even begin by debating the title which was chosen by our committee: «Contact dermatitis versus atopic eczema.» I personally would have preferred: «Contact eczema versus atopic dermatitis.» The term «atopic derma-

On the contrary, we did not find any reaction to the following antigens:

1. an antigen characteristic of duck eggs;
2. an antigen characteristic of duck meat.

It appears then, that there is no idiosyncrasy towards those antigens with which the possibility of previous contact can be entirely excluded, namely, the antigen characteristic of duck eggs, and the antigen found in duck meat.

Furthermore, 30 years ago, it was shown by *Dale* and *Hartley*, and *Dakin* and *Dale*, that guinea-pigs sensitized to hen egg-white also reacted to duck egg-white, and vice versa. We were able to demonstrate that guinea-pigs sensitized to hen egg-white, and examined by the *Schulz-Dale* method, reacted to exactly the same antigens as the infant who is idiosyncratic to egg-white. It would seem therefore, with a probability which approaches certainty, that the idiosyncrasy of the eczematous child is an acquired one, and acquired by sensitization. *Moro* had already supposed this, although without proof, and had suggested that it could be avoided by keeping the child from all contact with egg-white, even as early as the pre-natal period.

The question arises as to whether this idiosyncrasy to egg-white has an etiological significance in this dermatosis of small children Many authors, among whom *Moro* was the first, appear never to have had any doubts upon the subject; and yet, in 1926 (several years before *Moro*'s work), I had described the following case of idiosyncrasy toward hen egg. With a little *hen egg*, we were able to set off, by scarification, an urticarial reaction in a child, and obtain a positive *Prausnitz-Kustner* reaction with his serum. One year before these experiments, the child, who was two years old at the time, had been hospitalized for infantile eczema. He had been given egg to eat, and this had provoked urticaria, but without aggravation of his eczema. In later years, following the publication of the work of *Moro* and his colleagues, we frequently tried, in Bruno Bloch's clinic, to find a distinction [illegible]

[illegible] Perhaps at first sight one might be led to answer in the affirmative. The eczematous child shows a cutaneous reaction to egg which non-eczematous children do not; they all probably receive some egg in their feedings, either directly, or indirectly

of infantile neurodermatitis rather than infantile eczema; or, if we wish to reserve the word neurodermatitis for older children and adults, we speak, as *Rost* said, of früh-exudatives Ekzematoïd, or of atopic dermatitis of small children. But I am not so optimistic as to imagine that one can thus eliminate terms which have been employed for such a long time.

It would not be within the proper scope of this lecture, were I to speak to you at great length today of infantile eczema. I have already stressed its relation with the other atopies, if I may be permitted to use such an expression.

The test for contact eczema is the patch-test, which has not given really significant results with infantile eczema. I have had no personal experience with the patch-tests of *Simon*, performed with human dander on cases of infantile eczema. On the other hand, there are scratch tests and intradermal tests which have demonstrated that in infantile eczema one can obtain urticarial reactions, especially with hen egg-white. This was shown independently by *Rackemann* and *Moro*. This hypersensitiveness to egg-white has been successfully transferred by the *Prausnitz-Küstner* method. Thus, in infantile eczema, a condition in which heredity plays such an important role, we very frequently find an idiosyncrasy toward hen egg-white. Let us consider for a moment this allergic reaction. It raises certain problems, for example, is this reaction due to a sensitization? Certain authors have denied it. *Woringer* emphasizes that he found this idiosyncrasy in children who certainly had had no contact with hen eggs, the mother having already quite strictly avoided them throughout pregnancy. Similarly, *Buchs* and others have denied sensitization, for the reaction can be set off not only with hen eggs, but with other eggs with which there was not the slightest possibility of any previous contact. However, my collaborators, *Schaaf*, *Golay* and *Brun*, and I have come to the conclusion that, with a probability which approaches certainty, the idiosyncrasy which the atopic infant shows towards egg-white is due to a sensitization. With the *Prausnitz-Kustner* method, we have been able to demonstrate that the child reacts to the following antigens:

1. an antigen common to hen eggs and duck eggs;
2. an antigen found only in hen eggs;
3. an antigen found in hen eggs and chicken meat.

allergy, and its inclusion in the clinical management of these patients, will be rewarded by much more success in treatment than has previously been obtained.»

I should like to cite something which to my mind has always seemed to be an argument in favour of the importance of dust in neurodermatitis I am referring to the observation that, during the period of their military service, men with neurodermatitis show exacerbations when they are required to sleep on straw. Another fact which seems to speak clearly in favour of the importance of inhalants, is that a change of environment may undeniably have a favourable influence upon cases of atopic dermatitis. A stay at the seashore has often been especially recommended; and I believe that Swiss dermatologists have all observed many extraordinary *remissions* during a stay in the mountains, particularly in the St. Moritz area. These remissions are almost always followed by relapse when the patient returns to lower-lying regions. Furthermore, *Storm van Leeuwen* demonstrated with asthmatics that house-dust from St. Moritz and from Arosa caused fewer reactions than that of Basel and of Vulpera. In November of 1953, my collaborator, *Musso*, extracted an antigen from house-dust collected in a Geneva [illegible]

[illegible]

[illegible] from neurodermatitis, and on controls; he found no difference between the Geneva antigen and the St. Moritz antigen, even when he employed them in various dilutions. The same result was obtained by comparing another Geneva antigen with another St Moritz antigen, these two having been prepared from house-dust collected in the same schools but in February 1954. These experiments seem to argue against the hypothesis that a change of quality of house-dust is responsible for the improvement seen at St. Moritz in patients with neurodermatitis. While it is obvious that these experiments alone are not sufficient to exclude the possibility that house-dust plays an important role in the pathology of neurodermatitis, nevertheless, they do cast some doubt upon the validity of such an assumption. However, contrary to our results, *Schuppli* found that dust from the St-Moritz area caused many fewer reactions than that of Basel and of Zurich; but let us note that *Schuppli*'s experiments were made with asthmatics, and that he employed mattress-dust. With house-dust from the floor, such as we used, he obtained only few positive reactions

via the mother's milk. But it must be emphasized that the reaction set off in the skin by the application of egg-white is an urticarial reaction, while the dermatosis is eczematoid.

Leaving behind us now, at least for the time being, these few remarks concerning the atopic dermatitis of small children, let us consider for a moment the atopic dermatitis of their elders. The clinical picture is rather different. As a rule, the condition is less acute. Lichenification occurs, and the front of the elbow and back of the knee are especially involved. But the clinical diagnosis is frequently not sufficient, and we must take heredity, the case history, and the progress of the condition into consideration.

The reaction towards egg-white, which facilitates the diagnosis of infantile eczema, is no longer positive at the stage which we have taken the habit, perhaps a bad habit, of calling neurodermatitis. But there are other urticarial reactions which can often be obtained – reactions toward foodstuffs, and above all, reactions with inhalants, such as pollens, molds, dander, silks, and especially house-dust, in other words, the substances employed in the diagnosis of bronchial asthma. But although these reactions are so frequently positive, can we maintain that they are etiologically significant in this dermatosis? It is interesting to discover how widely the opinions of different authors vary with respect to this problem. Indeed, it is almost amusing to compare, for example, the articles by *Rostenberg* and by *Tuft* on this subject, both of which were published in 1955. The first wrote: «Persons with *this disease often display multiple positive immediate wheal reac*tions to a variety of protein allergens. They may have transference antibodies to some or all of the substances which yield wheals on direct testing. Occasionally, but quite rarely, the lesions have exacerbated when the person was exposed to certain allergens. Occasionally, but quite rarely, the lesions are improved by avoidance of, or desensitization to, specific allergens.»

Tuft, on the contrary, describing his experiences with children up to 12 years of age, reported: «Inhalant allergens, especially house-dust, plant pollens, atmosphere molds, and wood, can be just as important excitants of atopic dermatitis in children as of asthma. Failure to appreciate this has been responsible for the seeming intractability of some of these patients. Whereas their detection and correction often will be followed by noticeable and lasting improvement, consideration of the importance of inhalant

dency to overlook the distinction between these two types of reaction, which is, after all, rather striking. I still remember how, as a young assistant to *Bruno Bloch* in 1926-27, I tried to set off an urticarial reaction in a case of eczema to aloe, and in a case of eczema to primrose, but although the patch-tests were strongly positive, I was unable to obtain the slightest wheal. I can cite two cases of idiosyncrasy to camomile, which *Zaruski* and I published. The first was urticaria to camomile; the scratch-test yielded a very strong "immediate" reaction, but the patch-test was negative. The other case was an eczema to camomile. Here, the patch-test was positive, while the scratch-test was completely negative. To my mind, the difference between these two reactions has not been sufficiently appreciated until recently, otherwise it would not have been possible to simply assume that histamine, or a histamine-like substance, could be responsible for these two reactions—histamine, with which no one has ever obtained an eczematous reaction, never a delayed reaction.

If we have a case of eczema, probably contact eczema, we try to find the substance which provoked it. We do patch-tests with those substances which we consider suspicious in the light of the case history; these are what I have preferred to call "tests dirigés" ("oriented tests"). But one can also do patch-tests with a standard series of substances which are known to frequently cause eczema. Many years ago, *Bloch* and *Jaeger* already pointed out that, aside from monovalent eczematous patients, there are also polyvalent eczematous patients, that is, patients with eczema who react to a variety of substances. If, however, a standard series of patch-tests is performed with patients suffering from neurodermatitis, it soon becomes evident that here positive reactions are rare. In other words, a polyvalent eczematous sensitivity is much more frequent in contact eczema than in atopic dermatitis.

The patch-
and histological
it be allergic or
local infiltration
both intra- and … Thus, this histological picture is quite different from that of contact eczema, as well as of atopic dermatitis. I shall not enter upon a discussion of the histology of infantile eczema, of neurodermatitis, and of contact eczema (Prof. *Businco* will speak to us on this), but I should like merely to cite here two

with his asthmatics, while *Musso* obtained many positive reactions with adults suffering from atopic dermatitis, but few with the controls. *Schnyder*, from Miescher's clinic, is rather sceptical with regard to the role played by the various inhalants in neurodermatitis, for he only exceptionally found a positive reaction to inhalants in cases of neurodermatitis which were not accompanied by asthma or allergic rhinitis.

It appears, therefore, that we should not attach too much importance to urticarial reactions to various «inhalants». When I spoke of the egg-white reaction in infantile eczema, I explained that an urticarial reaction can be found in an atopic dermatitis without having an etiological significance. It seems to me that there is a very striking relationship between the reactions to egg-white and the reactions to inhalants. I admit that it is perhaps not very agreeable to relegate these reactions to the position of having no importance other than that of a diagnostic aid, but I should like to stress again the point that, in adults as well as in infants, atopic dermatitis is not urticaria, and the urticarial reactions which we obtain do not therefore correspond either clinically or histologically to the dermatoses.

I should like now to speak to you of contact eczema, and, as is my duty, I shall compare it with atopic dermatitis, often referring to my foregoing remarks, which I shall occasionally try to complete. Some of our colleagues may perhaps have the impression that heredity plays the same role in contact eczema as in atopic dermatitis; others may perhaps be of the opinion that the two conditions have the same relationship to asthma and hay fever. I would suggest that they proceed as my collaborators and I have done for years. They should choose cases in which the diagnosis is certain, and I am sure that they will then soon realize that the two conditions are clearly different in these respects. In contact eczema, heredity appears to play a small role, if indeed any. Furthermore it is usually not possible to demonstrate any relationship of contact eczema to asthma and hay fever. I will not speak of special cases such as eczema and asthma to ursol, since *R. L. Mayer*, the specialist in this field, is among us today.

We have already noted that the urticarial reaction is typical of atopic dermatitis (although this does not imply an etiological relationship). In contact eczema, the eczematous reaction is typical. I am somewhat surprised that there has been such a widespread ten-

The practical value of these tests seems to me to be undeniable, and I know of no comparable procedure with regard to atopic dermatitis.

The urticarial reaction of the infant with atopic dermatitis to egg-white, and of older children and adults to various "inhalants", can be transferred by the *Prausnitz-Kustner* method. This method however has failed so completely with contact eczema, that the question has arisen as to whether the eczematous reaction is truly due to an antigen-antibody reaction. Thanks to the work of *Landsteiner* and *Chase*, important progress has been accomplished in this field. *Landsteiner* and *Chase*, and later *Chase*, and also many others, investigated the passive transfer of eczematous hypersensitivity, not in humans however, but in guinea-pigs with contact eczema. Before speaking of the results of this passive transfer, allow me to say a few words concerning experimental eczema in guinea-pigs, which was first introduced to experimental dermatology by my former chief, *Bruno Bloch*. Unfortunately, we know of no cases of animal atopic dermatitis, but an experimental contact eczema can be produced in guinea-pigs. The only problem here, is whether we may properly permit ourselves to equate human contact eczema with that of guinea-pigs. I will not enumerate to you all the analogies between the two conditions, but I should like to cite an objection to considering [illegible]

[illegible] of eczema in guinea-pigs, *Sulzberger* considered as doubtful the validity of speaking of true animal contact eczema. At the time of their early experiments, *Bloch* and *R. L. Mayer* had already emphasised that the lesions obtained in guinea-pigs with primrose and with ursol histologically approximately correspond to human contact eczema. We were able to demonstrate that guinea-pig eczema completely corresponds to human eczema, if it is provoked not on the flank, but on the nipple, which has a thicker epidermis, and where there are no interfering hairs. Later, in *Sulzberger*'s laboratory, and in mine, the same result was obtained, namely a histologically typical eczema on the flank of guinea-pigs where we had thickened the epidermis. Thus, from the histological point of view, there is no objection to equating guinea-pig contact eczema with the human condition. Naturally, I do not mean to imply that the analogous, or let us simply say the identical histolo-

sentences from my father's writings (he did a good deal of work on this subject). Firstly – from the histological point of view, infantile eczema shows nothing which might distinguish it from adult eczema, and secondly, in disseminated neurodermatitis, we find the same lesions as in acute and chronic eczema.

We have seen that, with a probability approaching certainty, the urticarial egg-white reaction of infants with an atopic dermatitis is due to a sensitization. I have no opinion, one way or the other, as to whether this is also the case with the urticarial reactions of older children and adults suffering from neurodermatitis. As far as contact eczema is concerned, it is certain that sensitization plays a very important role. I even wonder whether there is such a thing as an idiosyncrasy without sensitization. *Darier* has given the following definition of idiosyncrasy: "A subject who reacts to a substance with which he has never yet had any contact, and which others tolerate with impunity, is considered idiosyncratic". Idiosyncrasy appears thus to be an inborn and constitutional sensitivity, but *Darier* continues, «these varieties are nearly superimposable on those due to sensitization. We may properly ask ourselves if it does not actually correspond to a previous sensitization, obscure, individual, or perhaps inherited». But let us put aside the problem of whether idiosyncrasies exist without sensitization, for one thing is quite certain, that is, different individuals are very differently sensitized, something which is particularly well demonstrated in the case of eczematous sensitivity. I must make another observation which, although terribly banal, seems to me to be nevertheless indispensable. A positive patch-test in a case of eczema which, in view of the case history and clinical picture, is probably a contact eczema, is obviously not a proof that the substance which yielded the patch-test was the true origin of the eczema. However, if this test has been carried out with a substance rendered suspicious by the case history, one can very frequently state, with a near-certain probability, that this substance did cause the eczema. On behalf of the Swiss National Accident Insurance Office, *H. Stauffer*, in 1929, and I myself, in 1947, investigated cases of eczema suspected of being occupational. In three-quarters of our cases, we found the origin of the eczema with a very high probability by applying patch-tests of those substances rendered suspicious by the case history. The Swiss National Accident Insurance Office recognized the validity of these tests, and the insurance was paid.

The practical value of these tests seems to me to be undeniable, and I know of no comparable procedure with regard to atopic dermatitis.

The urticarial reaction of the infant with atopic dermatitis to egg-white, and of older children and adults to various "inhalants", can be transferred by the *Prausnitz-Küstner* method. This method however has failed so completely with contact eczema, that the question has arisen as to whether the eczematous reaction is truly due to an antigen-antibody reaction. Thanks to the work of *Landsteiner* and *Chase*, important progress has been accomplished in this field. *Landsteiner* and *Chase*, and later *Chase*, and also many others, investigated the passive transfer of eczematous hypersensitivity, not in humans however, but in guinea-pigs with contact eczema. Before speaking of the results of this passive transfer, allow me to say a few words concerning experimental eczema in guinea-pigs, which was first introduced to experimental dermatology by my former chief, *Bruno Bloch*. Unfortunately, we know of no cases of animal atopic dermatitis, but an experimental contact eczema can be produced in guinea-pigs The only problem here, is whether we may properly permit ourselves to equate human contact eczema with that of guinea-pigs. I will not enumerate to you all the analogies between the two conditions, but I should like to cite an objection to considering that they are identical, that is, the histological difference between human eczema und guinea-pig eczema. In view of the difficulty of demonstrating histological lesions typical of eczema in guinea-pigs, *Sulzberger* considered as doubtful the validity of speaking of true animal contact eczema. At the time of their early experiments, *Bloch* and *R. L. Mayer* had already emphasised that the lesions obtained in guinea-pigs with primrose and with ursol histologically approximately correspond to human contact eczema. We were able to demonstrate that [illegible]

[illegible] hairs. Later, in *Sulzberger*'s laboratory, and in mine, the same result was obtained, namely a histologically typical eczema on the flank of guinea-pigs where we had thickened the epidermis. Thus, from the histological point of view, there is no objection to equating guinea-pig contact eczema with the human condition. Naturally, I do not mean to imply that the analogous, or let us simply say the identical histolo-

gical picture is alone a sufficient proof that the two conditions are identical. I have mentioned that, according to my father, there is no positive histological difference among infantile eczema, neurodermatitis and contact eczema; but my father would have strongly objected to assuming from this that these three conditions are identical. Surely we all agree that histology alone does not permit us to identify diseases; let us simply recall the case of tuberculoid structures.

As *Landsteiner* and *Chase* have demonstrated, by quite different methods, animals with contact eczema to DNCB or to picryl chloride may show, not only an eczematous sensitivity, but also a sensitivity of the anaphylactic type. This latter sensitivity can be passively transferred with serum, and with a modified *Prausnitz-Kustner* method, an urticarial reaction can even be obtained in the receiving animal. In 1926, I found a comparable phenomenon in a child who showed urticaria to honey. Apart from the urticarial reaction to honey, transferable by the *Prausnitz-Küstner* method, there was also a weak, but definite, eczematous reaction set off by the patch-test. I have rarely seen in man this combination of eczematous reaction and urticarial reaction. According to *Landsteiner* and *Chase*, there are many observations in guinea-pigs which indicate that we should clearly differentiate the anaphylactic reaction from the eczematous reaction.

From the experiments of *Landsteiner* and *Chase*, and from the experiments of those who repeated and modified their work, it has been demonstrated that the eczematous sensitivity can be transferred by cells from the peritoneal fluid, from lymph glands, from the spleen, or from the blood, so that one can obtain a delayed reaction in the receiving animal, a reaction apparently completely similar to that obtained in the donors by epicutaneous application of the substance responsible for the eczema. *De Weck* and *Brun*, in my laboratory, were able to confirm passive transfer in contact eczema to picryl chloride. They obtained strong reactions to picryl chloride in animals to whom they had previously made an injection of cells from the spleen of donors sensitized to this chemical.

However, there is a new theoretical difficulty which the work of my collaborators has brought to light. The reaction of the animal receiving the injection, which clinically resembles the donor's reaction, shows no histological eczematous changes. It seems to

me that these theoretically significant experiments should be pursued further, but having cited them, I will not discuss them at greater length.

Let us now return, for the last time, to a comparison of atopic dermatitis with contact eczema.

Cortisone is certainly not a substance which we may simply call anti-inflammatory. This has been clearly shown by the cooperative research accomplished by three Geneva institutes, the Ophthalmological Clinic, the Institute of Histology, and the Dermatological Clinic. The following conditions were found to be unaffected by cortisone: herpes simplex of the cornea, the tuberculin reaction in the bovine conjunctiva, the reaction of guinea-pigs to Schick toxin and to cantharidine, and the reaction in humans to ultraviolet irradiation.

It cannot be overemphasized that atopic dermatitis and contact eczema respond impressively to this hormone, although relapses are very common after cessation of treatment, especially in cases of atopic dermatitis. But, and this we consider to be noteworthy, according to our experiments (*Franceschetti* and his collaborators), the urticarial reactions, which are characteristic of atopic dermatitis, are not abolished by cortisone. With regard to the patch-tests, the situation is not as clear, for some investigators, such as *Ingram* and his collaborators, have seen them become negative under the influence of cortisone therapy, while others, such as *Sulzberger* and his collaborators, have not been able to confirm this.

And so we are confronted with still another enigma in a field which is so complex, so full of enigmas, the solution of which will require reflection and discussion, but more important than reflection is further observation and experimentation. We are still very far from a conclusion, and this will be my principal conclusion, apart from the following rather obvious one: The differences between atopic dermatitis and contact eczema are great, and the fact that the differential diagnosis may be very difficult is not a proof that the two conditions are related.

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Busınco, L., Benvignatı S., et Avanzıni, F. Int. Arch. Allergy 11 32—43 (1957)

Dermatite allergique expérimentale par chlorpromazine et action inhibitrice du goudron végétal

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La chlorpromazine cause dans la peau des réactions d'hypersensibilité, caractérisées surtout par un œdème le plus souvent imposant et dont l'apparition est rapide. On peut reproduire, dans les expériences, cette hypersensibilité sur la peau des cobayes. Nous avons en outre envisagé la possibilité d'agir contre le développement de cette réaction au moyen d'applications d'une mélange de goudron végétal (dévalile).

La recherche expérimentale a été faite sur trois groupes de cobayes.

Le premier groupe a été traité tous les jours avec des applications cutanées d'une solution aqueuse de chlorpromazine à 2,5 %. Une partie des cobayes a été sacrifiée après 5 et après 10 jours du traitement. Les autres cobayes sont restés en repos pendant 10 jours, ensuite on a sacrifié trois animaux, tandis que l'on a appliqué la chlorpromazine encore une fois aux autres. Ces derniers animaux ont été tués en partie au 22e jour, en partie au 23e et encore au 24e jour.

Fig 1. Peau de cobaye 5 jours après l'application cutanée de chlorpromazine tous les jours Epiderme et derme à structure normale

Fig 2 Idem Détail du derme normal

Fig 3 Idem Le tissu conjonctif du derme réticulaire paraît profondement œdémateux Les fasceaux sont mal colorés et espacés l'un de l'autre

Fig 4 Idem Groupe des vaisseaux du derme réticulaire On remarque une nette ectasie atteignant surtout les capillaires

Fig 5 Idem Par un agrandissement plus fort, on remarque une dissociation œdémateuse des fibres conjonctivales dans le derme papillaire On voit aussi une remarquable ectasie des anses capillaires du réseau vasculaire superficiel

Fig 6 Idem Detail des capillaires ectasiques Ils paraissent remarquablement dilatés et vide de sang

Fig 7 Idem Détail de l'œdème atteignant sérieusement le tissu conjonctif du derme profond

Fig 8 Idem Epiderme avec des plaques de spongiose circonscrite

Fig 9 Peau de cobaye traitée pendant 5 jours avec un mélange de goudron Hyperkératose moderée avec des signes de dégénération de l'épiderme. La couche cornéenne montre une infiltration leucocytaire Signes modérés d'infiltration dans le derme papillaire.

Fig 10 Idem Detail, le conjonctif papillaire paraît œdémateux et désagregé avec des signes de souffrance dégénérative

Fig 11 Idem. Detail La couche germinative montre une activité reproductive évidente avec caryocinese

Fig 12 Idem Vue d'ensemble de la peau avec les signes d'hyperkératose et épaississement de la couche germinative Aspect normal du derme

Fig 13 Idem On remarque un enrichissement sensible des cellules de la couche germinative. Oedeme du tissu conjonctif contigu

Fig 14 Peau de cobaye traitee pendant 10 jours avec un mélange de goudron, Repos pendant les dix jours consécutifs Nouvelle application du mélange au 21e jour. L'animal est sacrifié au 23e jour Couche épidermique épaissie par prolifération de la couche muqueuse de Malpighi.

Fig 15 Idem Detail de l'enrichissement fibroblastique dans les interstices du derme profond

Fig 16 Peau de cobaye traitee chaque jour avec un mélange de goudron et Chlorpromazine pendant 10 jours Ensuite repos pendant 10 jours, sacrifice de l'animal au 21e jour Peau d'aspect à peu pres normal avec des signes modérés d'hyperplasie épidermique

Fig 17 Peau de cobaye traitée chaque jour avec un mélange de goudron et Chlorpromazine pendant 10 jours Ensuite repos pendant 10 jours, nouvelle application du melange au 21e jour Animal sacrifié au 23e jour Hyperkèratose, épaississement de la couche de Malpighi, infiltration du derme papillaire. Dans le derme profond il y a un œdeme moderé et une augmentation des fibroblastes

Fig 18 Idem Detail [illegible]

Fig 19 Peau de col [illegible]
goudron et Chlorprc [illegible] ensuite repos de 10 jours [illegible]
une nouvelle applica[illegible]
keratose, épaississem[illegible]
Oedeme [illegible]

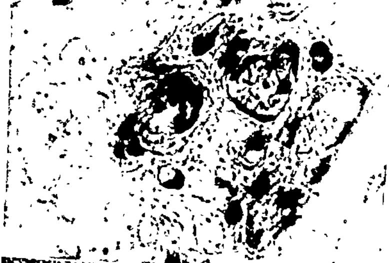
Fig 20 Idem Anı[illegible]

Fig 21 Idem Oedeme du conjonctif papillaire avec l'ectasie des capillaires du réseau superficiel



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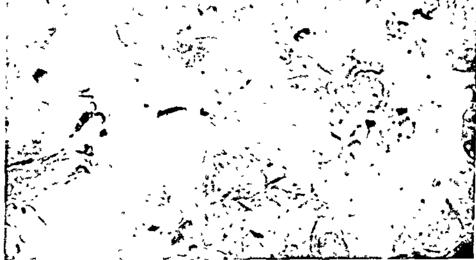




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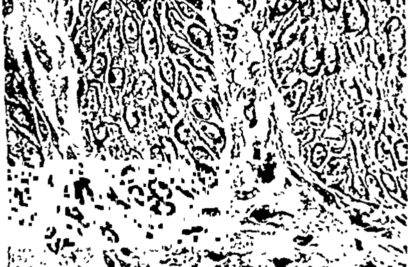


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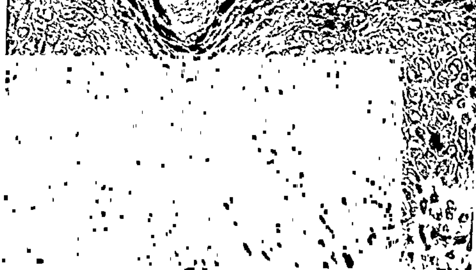


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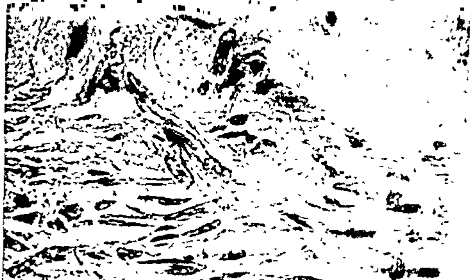


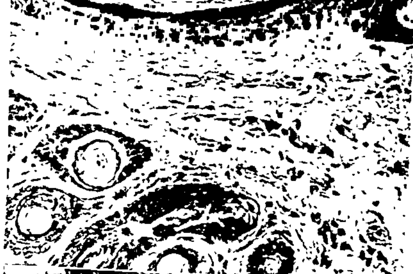


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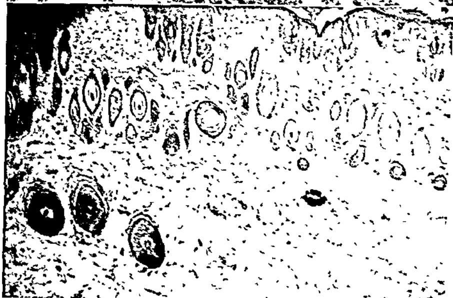


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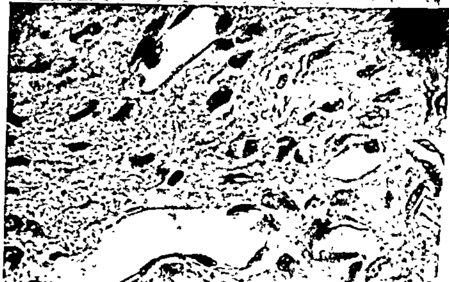




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Le deuxième groupe a été traité tous les jours avec des applications cutanées d'un mélange de goudron végétal et les cobayes ont été sacrifiés en partie après le 5e jour et les autres après le 10e jour du traitement. D'autres encore sont restés en repos pendant 10 jours, ensuite on a tué 3 animaux, tandis qu'aux autres on a appliqué encore une fois le mélange de goudron; on les a tués après le 22e, 23e et 24e jour.

Le troisième groupe a été traité avec le mélange de goudron et la solution de chlorpromazine à 2,5 %; les animaux ont été sacrifiés aux mêmes laps de temps que les animaux des autres groupes. Dans cette expérience on a introduit une variation, c'est-à-dire on a fait, pendant 10 jours de suite, un traitement avec la solution de chlorpromazine à elle seule et, dans les jours suivants, des applications quotidiennes de goudron végétal et ensuite, au 21e jour, on a badigeonné de nouveau avec la solution de chlorpromazine. On a sacrifié ces animaux de la même manière que les autres animaux.

Nous rapportons ici les résultats des examens *histologiques* de la peau des animaux traités avec la chlorpromazine, avec le mélange de goudron et encore avec la chlorpromazine associée au mélange de goudron végétal chez les mêmes animaux.

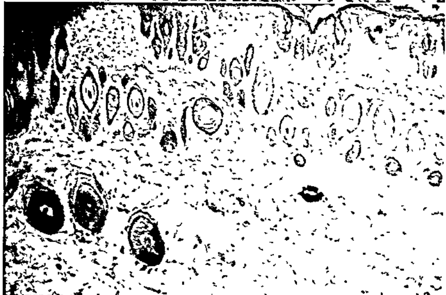
Résultats

Les examens histologiques faits dans nos expériences nous permettent d'établir les manifestations fondamentales suivantes.

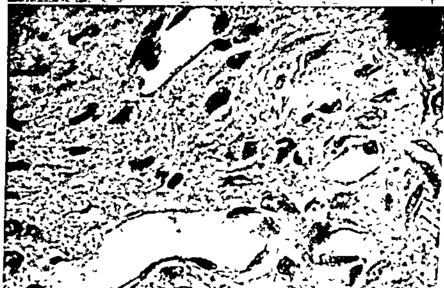
Le *traitement* avec la solution de chlorpromazine à 2,5 % cause, dans la peau des cobayes, une réaction d'hypersensibilité localisée surtout dans le derme profond ou réticulaire. L'œdème y est d'une intensité remarquable et il dissocie violemment les gros faisceaux conjonctivaux en reduisant leur colorabilité. Une souffrance œdémateuse coexiste dans les couches superficielles où l'on observe une grave ectasie des capillaires. L'œdème atteint même la couche basilaire de l'épithélium et cause la *spongiose*. Dans les peaux traitées avec la chlorpromazine, l'intensité de l'œdème contraste avec l'absence totale de la réaction proliférative des histiocytes périvasculaires et avec le manque d'infiltration de granulocytes éosinophiles et d'autres cellules que l'on rencontre ordinairement dans l'inflammation allergique. La souffrance du tissu est la manifestation d'un trouble grave de la perméabilité capillaire avec épanchement copieux de liquide, dont le contenu protéique est pauvre.



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rait même dériver d'une action analgésique locale. Il faut encore considérer l'hypothèse d'une action irritante sur les fibres nerveuses, causée par la crise hyperergique et qui, moyennant des processus de vaso-dilatation antidromique et des axone-réflexes, etc., augmente la libération anormale locale de l'histamine. Le fait qu'à cette libération de l'histamine ne s'ajoute pas la présence des éosinophiles, pourrait être expliqué par une action d'inhibition de la part de la chlorpromazine envers ces cellules. Il ne faut pas exclure enfin que l'œdème par chlorpromazine puisse avoir lieu, dans les crises d'hypersensibilité, au moyen d'un agent chimique qui ne soit pas l'histamine.

Quant à l'action contrastante du mélange de goudron végétal contre la réaction hyperergique cutanée à la chlorpromazine, elle pourrait être expliquée soit par une simple inhibition au contact complet avec l'allergène, soit par un mécanisme biologique plus complexe. Dans ce dernier l'augmentation des fibroblastes peut avoir son importance et aussi les activités biologiques opposées, provoquées par le mélange goudronneux lui même.

Résumé

Le traitement avec la solution de chlorpromazine a 2,5 % cause, dans la peau des cobayes, une réaction d'hypersensibilité localisée surtout dans le derme profond ou réticulaire. L'œdeme y est d'une intensité remarquable et il dissocie violemment les gros faisceaux conjonctivaux en reduisant leur colorabilité.

Une souffrance œdémateuse coexiste dans les couches superficielles ou l'on observe une grave ectasie des capillaires. L'œdème atteint même la couche basilaire de l'épithélium et cause la spongiose. L'application d'un extrait de goudron végétal peut inhiber cette réaction.

Summary

Application of a 2.5 % solution of chlorpromazine evokes in the sk[illegible]
a [illegible]
de[illegible]
co[illegible] [illegible]ability.

An oedematous state also exists in the superficial layers where one observes a severe ectasis of the capillaries. The oedema even reaches the basilar epithelial layer and causes spongiosis. The application of a vegetable tar extract inhibits this reaction.

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Le traitement avec le mélange de goudron végétal à lui seul cause, dans la peau des cobayes, une réaction bien évidente atteignant toutes les couches. La couche cornée est considérablement épaissie et infiltrée par les leucocytes. La couche muqueuse a augmenté son épaisseur et s'enfonce par de grosses branches dans le conjonctif dermique, car elle est riche en caryocinèse dans sa partie basilaire. Le conjonctif prend l'aspect œdémateux, il est parsemé de cellules phlogistiques, mais il montre surtout une nette prolifération de fibroblastes, ce qui est bien évident dans le conjonctif dermique profond, tandis qu'à l'ordinaire il en est très pauvre.

Le traitement avec la solution de chlorpromazine et du mélange de goudron chez les mêmes animaux a démontré, à l'examen histologique, une réduction variable de la réactivité œdémateuse hyperergique. Dans la peau des animaux de ce groupe, la réaction au mélange goudronneux s'oppose nettement au développement de la réaction œdémateuse causée par la chlorpromazine. La manière dont ce contraste a lieu peut avoir de différentes interprétations.

De ces observations histologiques, on peut tirer des conclusions. Il faut considérer d'abord le caractère exclusivement œdémateux de la réaction d'hypersensibilité cutanée à la chlorpromazine, réaction qui est soutenue par une très grande ectasie des capillaires et par le trouble conséquent de la perméabilité, comme nous avons déjà dit. Bien que le caractère d'une réponse hyperergique soit bien clair, il faut remarquer l'absence de ces mouvements de la réaction histiocytaire qui partent de la paroi des petits vaisseaux, et aussi le manque des éosinophiles. En effet les réactions histiocitaires périvasculaires ont lieu à l'ordinaire dans toutes les inflammations allergiques; en outre la présence des éosinophiles est un signe bien caractéristique des réactions tissulaires où il y a la libération de l'histamine. Tandis qu'ici il y a, en effet, le tableau réactif que l'on observe dans les œdèmes angioneurotiques, dont la pathogénèse, selon le type classique de l'allergie, n'est pas encore certaine. Dans l'hypersensibilité causée par la chlorpromazine, l'altération de la perméabilité capillaire est probablement provoquée en grande partie par un mécanisme nerveux jouant un rôle dans la réaction antigène-anticorps. La manière dont ce phénomène a lieu n'est pas encore éclaircie. On peut envisager l'hypothèse d'une action locale de la chlorpromazine, qui par l'inhibition du nerf sympathique contribue à augmenter la vaso-dilatation des capillaires, causée par la réaction d'hypersensibilité L'accentuation de l'ectasie capillaire pour-

stances protéiques. Même les nucléus des capillaires sont grossis à cause de l'augmentation de la part aqueuse. L'examen, fait en détail à fort grossissement, permet de déceler des bulles et des boutons minuscules dans la paroi tout autour du capillaire, signe d'un état d'hyperactivité de passage du corps fluide du capillaire dans le tissu environnant. A ce tableau morphologique correspond une augmentation anormale de la perméabilité, par conséquent les tissus contigus deviennent très riches d'un liquide aqueux. L'intensité de ce phénomène est démontré par le fait que tout près des capillaires il y a fréquemment des lacunes dissociant l'intégrité des tissus. L'examen histologique a démontré que de ces lacunes partent de minuscules courants de liquide, qui vont dissocier les cellules de la couche muqueuse adjacente et surtout celles de la couche basilaire. Cette irradiation fluide, partant du capillaire de la papille du derme, prend souvent un aspect arboriforme *caractéristique*, car elle diminue de relief au fur et à mesure qu'elle *s'éloigne vers* la partie extérieure de l'épithélium.

Sur la base de ces observations, on remarque que l'altération fondamentale est constituée par une souffrance des capillaires avec *l'augmentation* anormale de leur perméabilité et conséquente imbibition séreuse et œdème des tissus adjacents, et aussi de troubles *différents de l'intégrité* structurale des mêmes tissus.

Outre les altérations dont la *nature est surtout* fonctionnelle, les capillaires peuvent montrer aussi d'autres *transformations* morphologiques consécutives représentées surtout par une augmentation *des cellules* histiocytaires près de leur *paroi*. Ces *histiocytes* – que nous appelons aristocytes – deviennent plus *nombreux* d'une manière variable sous la stimulation hyperergique et ils peuvent arriver à s'organiser en formations nodulaires périvasculaires Dans ces cas, on peut parfois trouver de nombreux granulocytes éosinophiles mêlés aux histiocytes, ce qui est *un autre signe* de la réaction allergique du tissu ayant lieu dans cette région vasculaire.

L'examen histologique de l'épithélium n'a pas décelé d'altérations aussi importantes. Il *faut* remarquer que les modifications morphologiques ont lieu *particulièrement* dans la couche basilaire et non dans la couche *superficielle*. Dans la couche basilaire, surtout à proximité du tissu conjonctif œdémateux, il y a des altérations avec spongiose et aussi un *renflement* d'intensité variable des cellules de l'épithélium Parfois il y a aussi, parallèlement à la

Businco, L., Granelli, U., Gaffi, A., et Centanni, G. Int Arch Allergy 11 44-60 (1957)

Le tableau histologique des dermatites hyperergiques par contact

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Dans ce travail nous avons soumis à la recherche histologique la peau de différents individus atteints de dermatite allergique par contact. Dans 5 cas d'hypersensibilité aux substances antibiotiques, nous avons examiné des fragments cutanés extirpés, au moyen de la biopsie, de la lésion hyperergique active. La même étude microscopique a été faite dans un cas d'hypersensibilité au D.D T. et au ciment en prélevant un fragment cutané dans une lésion érythémateuse spécifique. On a fait aussi une recherche histologique sur des fragments de peau prélevés dans le siège même de la réponse positive au test par contact vis-à-vis des allergènes représentés par pénicilline, bois de chêne.

Résultats

L'étude histologique de la peau, enlevée des lésions dermatiques causées par l'hypersensibilité au contact avec la penicilline et d'autres substances, nous a permis de faire les observations suivantes:

L'altération morphologique la plus importante, celle qui nous semble avoir une place prépondérante dans le déterminisme pathogénétique des différentes modifications cutanées, est formée par la souffrance des capillaires du derme papillaire, c'est-à-dire de la région située directement au dessous de la couche basilaire de l'épithélium. Ces capillaires sont les plus souvent ectasiques, leur calibre est très large et ils sont presque toujours sans les éléments morphologiques du sang. Leur endothélium est grossi à cause d'un processus d'imbibition par un liquide séreux très pauvre en sub-

de ces cellules, soit en passant par les voies glandulaires ou le long des voies piliphères, l'allergène atteint le derme papillaire et vient à contact avec les capillaires qui sont les éléments réactifs ultrasensibles à l'allergie. Ici l'allergène excite les histiocytes de la paroi capillaire, toujours prêts à élaborer les anticorps, et cause ainsi la sensibilisation locale. Dans ce siège, les contacts successifs avec l'allergène causent ensuite la libération des substances histaminiques dont la peau est particulièrement riche. Probablement c'est à

Fig 1 Cas 792 Eczéma par application locale de poudre de pénicilline. Oedème spongioïde du corps de Malpighi Le tissu conjonctif sous-épithélial paraît aussi œdémateux

Fig 2 Cas 792 Detail Par un fort agrandissement on voit un œdeme très intense du tissu conjonctif, dont les capillaires sont fortement ectasiques et vides de sang On remarque un relâchement de la contiguïté des cellules épineuses; les interstices cellulaires sont élargis et montrent leur continuation en des espaces interfibrillaires formés par l'œdeme dans le conjonctif

Fig 3 Cas 792 Autre detail Oedeme du tissu conjonctif avec souffrance spongiotique de la couche basale.

Fig 4 Cas 792. Detail Région d'une papille dermique avec un conjonctif fortement œdémateux On remarque quelque anses des capillaires très ectasiques L'œdeme a espacé le conjonctif adjacent et formé des lacunes interstitielles dont le liquide se répand dans l'épithélium de Malpighi en dissociant les cellules

Fig 5 Cas 793 Eczéma allergique par l'emploi local de pénicilline Oedème de la couche inférieure du corps de Malpighi Tissu conjonctif papillaire œdémateux avec des vaisseaux ectasiques et parfois modérément enrichi de cellules histiocytaires

Fig 6 Cas 793 Détail Derme papillaire œdémateux avec des vaisseaux ectasiques Le tissu conjonctif est espacé par des lacunes d'œdeme qui continuent dans les espaces intercellulaires abnormement enlargis du corps de Malpighi.

Fig 7 Cas 804 Dermatite allergique par emploi local de pénicilline L'épithélium est remarquablement épaissi, avec un fort processus d'acanthose Parmi des branches épithéliales il y a des anses du derme papillaire incluses, en différentes conditions de souffrance dégénérative.

Fig 8 Cas 804 Detail Les deux anses [illegible]

[illegible] dégénération.

Fig. 9 Cas 804 Détail Epithelium montrant l'inclusion de parties de [illegible]

Fig. 10 Cas 804 Dét. [illegible]
papillaires incluses

[illegible]

gravité de l'invasion séreuse, la dégénération de groupes de cellules épithéliales.

Quant à la formation des vésicules intra-épithéliales, l'examen histologique nous a permis d'observer une autre manière de formation, différente de l'enrichissement anormal primitif du liquide parmi les interstices épithéliaux. Quand l'épithélium, étant sous la stimulation de l'allergène, augmente d'épaisseur et s'enfonce dans le derme par de branches d'acanthose, il arrive que le tissu conjonctif du derme papillaire reste comme inclus dans les mêmes branches. Ce tissu conjonctif est nourri d'ordinaire par une anse des capillaires, qui à son tour est sous l'influence de la crise allergique et par conséquent est assujettie aux altérations susdites de la perméabilité et de la morphologie. Il s'ensuit que cette île de tissu conjonctif est envahie par l'œdème et qu'ainsi se forme une situation locale d'insuffisance métabolique. Elle est empirée par le fait que, comme la région conjonctivale est comprimée de tous côtés par l'épithélium, le mouvement régulier des liquides ne peut plus avoir lieu. Dans certains cas, l'étanchement de ces liquides, étant favorisé par la situation topographique de la papille conjonctivale pratiquement incluse parmi des parois plus rigides, cause la dégénération des éléments de la structure du conjonctif emprisonné, y compris les capillaires. La région ainsi endommagée se remplit de vacuoles œdémateuses finissant par confluer en formant ainsi des vésicules intra-épithéliales.

L'examen histologique des fragments cutanés, enlevés du siège des tests positifs envers les allergènes relatifs, a permis de confirmer l'importance pathogenétique prééminente de l'unité histiocapillaire dans le déterminisme des réactions hyperergiques de la peau. Ici même la souffrance primitive a le conjonctif du derme papillaire comme siège, et particulièrement les capillaires du réseau superficiel. Leur ectasie et l'altération de la perméabilité sont la cause de l'œdème dissociant la structure du tissu conjonctif et causant la spongiose épithéliale. En même temps, il y a la multiplication des cellules histiocytaires de la paroi qui tendent à la forme lympho-monocytoide. Il peut y avoir aussi une infiltration locale des éosinophiles

Quant à la pathogénèse des dermatites allergiques par contact, on peut envisager l'interprétation suivante sur la base des connaissances actuelles et des résultats de la recherche histologique: une fois surmontée la barrière épithéliale, soit au travers des interstices

de ces cellules, soit en passant par les voies glandulaires ou le long des voies pilifères, l'allergène atteint le derme papillaire et vient à contact avec les capillaires qui sont les éléments réactifs ultrasensibles à l'allergie. Ici l'allergène excite les histiocytes de la paroi capillaire, toujours prêts à élaborer les anticorps, et cause ainsi la sensibilisation locale. Dans ce siège, les contacts successifs avec l'allergène causent ensuite la libération des substances histaminiques dont la peau est particulièrement riche. Probablement c'est à

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Fig 7 Cas 804 Dermatite allergique par emploi local de

Cas 804 Detail Les deux anses conjonctivales adjacentes montrent des degrés differents de souffrance. Dans l'une on observe un vaisseau ectasique, avec paroi épaissie par l'œdeme, il s'enfonce dans le conjonctif œdémateux. Dans l'autre, le capillaire dans sa partie haute est désagrége et on y remarque la présence de cellules en differents états de dégénération.

Fig 9 Cas 804 Détail Epithelium montrant l'inclusion de

Fig 10 Cas 804 I
papillaires inclus

Fig 11 Cas 804
culaire partielle

Fig 12 Cas 806 Dermatite allergi
poudre Epithélium avec remarqu
dans sa base. Les vaisseaux du der
en partie granul

Fig 13 Cas 806 Détail Vaisseau du conjonctif papillaire avec un épaissement histiocytaire péripariétal

Fig 14 Cas 806. Détail Oedème péricapillaire dans le derme.

Fig 15 Cas 807 Dermatite allergique par emploi local de pénicilline en poudre L'épithélium paraît remarquablement épaissi avec une acanthose considérable La couche épithéliale est espacée par des plaques de conjonctif dermique incluses dans l'épaississement de l'épithélium Dans le derme, on remarque la présence de vaisseaux avec épaississement histiocytaire péripariétal

Fig 16 Cas 807 Détail Vaisseau du derme avec un épaississement histiocytaire, œdème du tissu conjonctif et présence d'éosinophiles

Fig 17 Cas 807 Détail Détail du différent aspect des plaques de conjonctif dermique incluses dans l'épithélium Quelques-unes montrent l'ectasie des anses capillaires et œdème du conjonctif, tandis que d'autres montrent les signes d'une souffrance dégénérative

Fig 18 Cas 807 Détail Souffrance de differents degrés des plaques conjonctivales incluses dans l'épithélium

Fig 19 Cas 807 Détail Région conjonctivale montrant un œdème considérable et transformation dégénérative de plusieurs éléments de la structure

Fig 20 Cas 796 Dermatite eczématiforme par D D T Vaisseaux du conjonctif dermique avec une réaction proliférative des histiocytes pariétaux

Fig 21 Cas 796 Détail Oedeme bien visible dans le conjonctif dermique avec augmentation des histiocytes périvasculaires

Fig 22 Cas 796 Détail Vaisseau du derme papillaire montrant une augmentation des histiocytes péripariétaux

Fig 23 Cas 796 Détail Ectasie capillaire dans le derme supérieur avec amassement des vaisseaux péripariétaux

Fig 24 Cas 796 Détail Hyperplasie histiocytaire péricapillaire avec des lacunes remplies de sérum

Fig 25 Cas 809 Eczéma allergique par ciment Epithélium de structure normale Oedème modére du conjonctif souseépithélial Vaisseaux avant les parois riches en cellules

Fig 26 Cas 809 Détail Dans la région du derme papillaire on observe un œdeme évident et aussi la présence de résidus nucléaires picnotiques et de fibroblases comme résultat d'une souffrance chronique

Fig 27 Cas 809 Détail Dans le derme on voit la présence de vaisseaux ayant les parois enrichis par des histiocytes évolus en cellules lymphomonocytoides Même les granulocytes neutrophiles y sont très fréquents

Fig 28 Cas 794 Hypersensibilité à la pénicilline (emploi local) Biopsie sur test cutané, après 36 heures Epithélium de structure normale Dans le derme, œdeme léger du conjonctif Quelques capillaires ectasiques avec presence de cellules histiocytaires et de nucléus endothéliaux

Fig 29 Cas 795 Hypersensibilité à la pénicilline (emploi local) Biopsie sur test cutané, apres 36 heures Epithélium avec des cellules hydropiques surtout dans la partie basilaire Le conjonctif est tres œdémateux avec ectasie vasculaire et augmentation des histiocytes péripariétaux

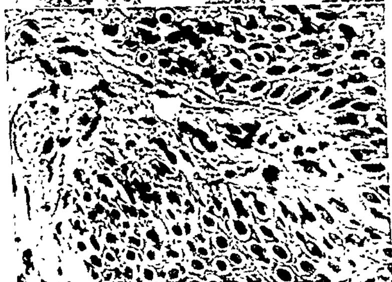
Fig 30 Cas 795 Oedeme et dégénération cellulaire

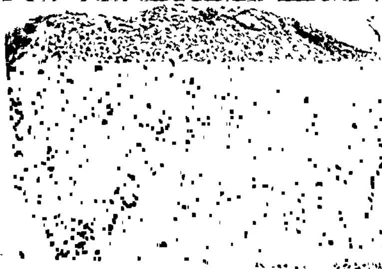
Fig 31 Cas 795 Détail Cellules épithéliales avec hydrope cytoplasmatique

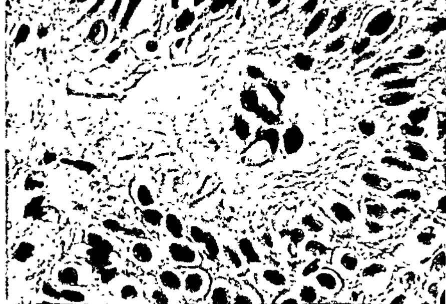
Fig 32 Cas 801 Hypersensibilité à la poussiere de bois de chêne Biopsie sur test cutané apres 72 heures Vésicules intra-épithéliales et œdeme du derme Il y a de nombreux vaisseaux avec amassement cellulaire periparietal

Fig 33 Cas 801 Autre detail Oedeme du conjonctif dermique avec des vaisseaux montrant un amassement de cellules dans la région pariétale

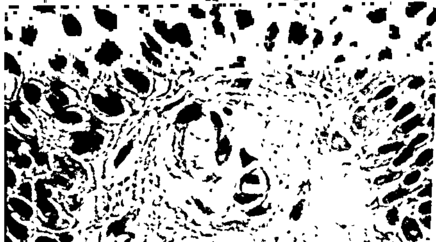

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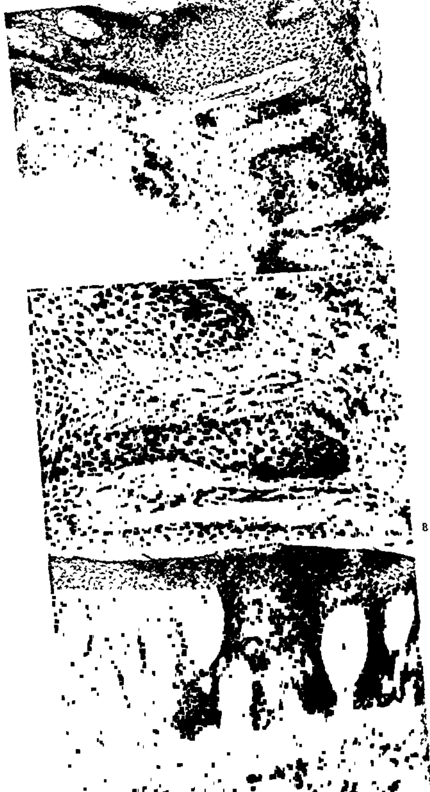

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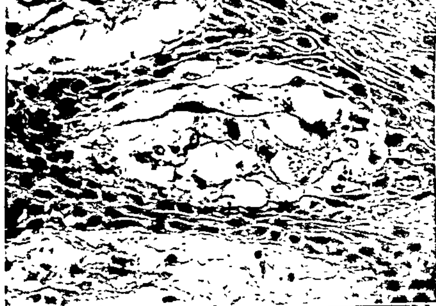

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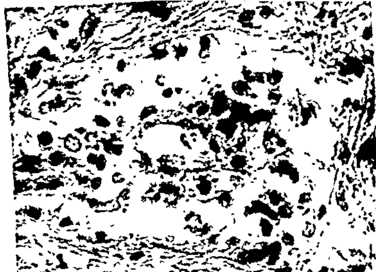


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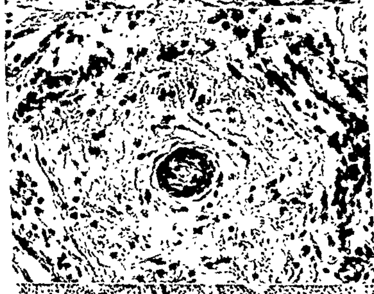


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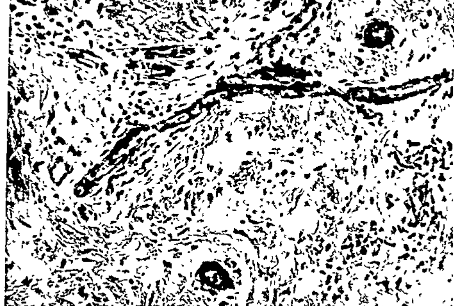




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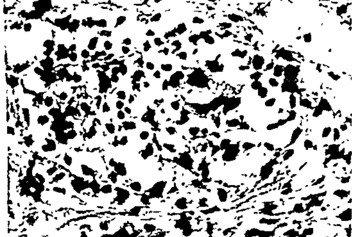


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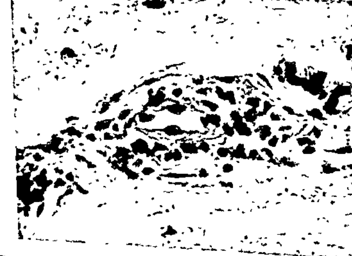


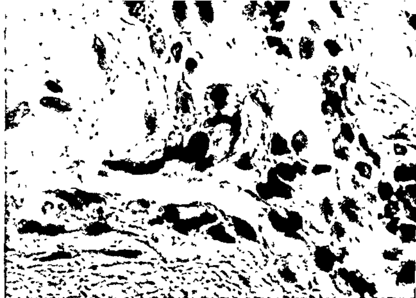
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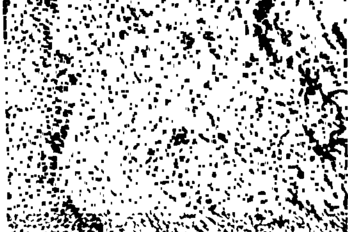

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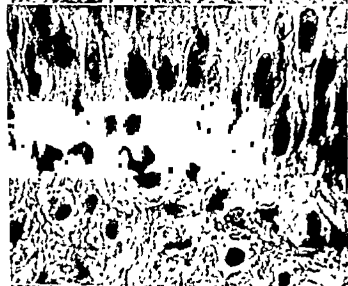

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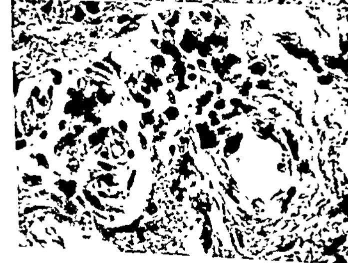


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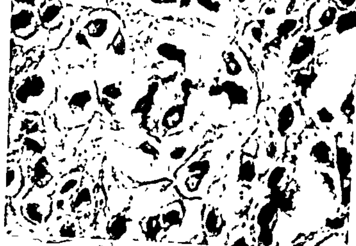

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l'histamine que l'on doit attribuer le rôle le plus important dans l'altération des capillaires et surtout dans l'augmentation morbide de leur perméabilité entraînant une imbibition séreuse plus ou moins remarquable des tissus adjacents. Cette intervention de l'histamine dans les manifestations de l'allergie est démontrée aussi par l'augmentation remarquable de l'histaminémie chez les individus porteurs d'eczéma, ce *que nous avons déjà remarqué en* 1941. La stimulation allergénique à comme conséquence aussi l'augmentation du nombre des cellules formant les anticorps, et à cause de cela on voit fréquemment que la paroi des capillaires est enrichie par de nombreux éléments à type lympho-monocytoïde provenant des histiocytes locaux, qui auparavant avaient été considérés comme des cellules infiltratives d'origine hématique.

Dans les cas où l'altération cutanée, causée par le contact direct avec l'allergène, est accompagnée par d'autres manifestations de la peau dans d'autres régions lointaines, il faut penser non seulement à un facteur prédisposant ayant une spécificité topographique, *mais aussi à un probable tropisme de* l'allergène pour les structures cutanées en général.

Résumé

Dans ce travail les auteurs ont soumis à la recherche histologique la peau de différents individus atteints de dermatite allergique par contact

Sur la base de ces observations, on remarque que l'altération fondamentale est constituée par une souffrance des capillaires, avec l'augmentation anormale de leur perméabilité et conséquente imbibition séreuse et œdème des tissus adjacents, et aussi des troubles *différents de l'intégrité* structurale des mêmes tissus

Outre les altérations dont la nature est surtout fonctionnelle, les capillaires peuvent montrer aussi d'autres transformations morphologiques consécutives, représentées surtout par une augmentation des cellules histiocytaires pres de leur paroi

Summary

In this paper the authors have investigated histologically the skin of various individuals suffering from allergic contact dermatitis

From these observations it appears that the fundamental process of change is represented by an affection of the capillaries in which there appears an abnormal increase *in permeability with consequent serous imbibition and oedema of adjacent* tissue and also various disturbances of the structural integrity of these tissues

In addition to these changes, which bear a predominantly functional character, the capillaries may also show other consecutive morphological transformations, as shown mainly by an increase in peripheral histiocytes

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Porter, R R Int Arch Allergy 11 61–63 (1957)

The Reagin Content of Human Gamma-Globulin Fractions

By R R PORTER

National Institute for Medical Research, London

Several attempts have been made to decide by electrophoretic techniques which fractions of the serum proteins contain the skin sensitising antibodies – the reagins. Zone electrophoresis (1) on starch and continuous electrophoresis (2) showed that the reagins were in the γ-globulin fraction while electrophoresis convection (3–5) suggested that they might be in any or all of the globulin fractions.

We decided to see if the greater resolution provided by the excellent chromatographic technique of *Peterson* and *Sober* (6) would give further information on this problem A full account of this work has appeared elsewhere (7).

The serum was fractionated on diethylamino ethyl cellulose by a slight modification of the technique of *Peterson* and *Sober* and a typical elution diagram together with details of the chromatography are given in Fig 1.

Assay of the reagins was by the *Prausnitz-Kustner* (8) passive sensitisation test and serum was obtained from members of the staff of this Institute who had a history of hay fever and/or asthma and who gave positive skin tests to pollen extracts, horse serum or cat or dog scurf extracts.

Results

When the chromatographic fractions shown in Fig. 1 were examined by [illegible] d [illegible] h [illegible] globulin. Fraction I was apparently a γ_2-globulin and the others γ_1-globulin. Fraction V moved at a rate between β and γ and later fractions had higher mobilities.

l'histamine que l'on doit attribuer le rôle le plus important dans l'altération des capillaires et surtout dans l'augmentation morbide de leur perméabilité entraînant une imbibition séreuse plus ou moins remarquable des tissus adjacents. Cette intervention de l'histamine dans les manifestations de l'allergie est démontrée aussi par l'augmentation remarquable de l'histaminémie chez les individus porteurs d'eczéma, ce que nous avons déjà remarqué en 1941. La stimulation allergénique à comme conséquence aussi l'augmentation du nombre des cellules formant les anticorps, et à cause de cela on voit fréquemment que la paroi des capillaires est enrichie par de nombreux éléments à type lympho-monocytoide provenant des histiocytes locaux, qui auparavant avaient été considérés comme des cellules infiltratives d'origine hématique.

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Table I
Reagin Activity of γ-Globulin Fractions
(Wheal area, in sq mm, with J M serum)

Concentration	Fraction 1	2	3	4	Euglobulin	Whole Serum
1/1	0	70	100	60	0	90
1/10	0	10	60	trace	0	80
1/100	0	0	trace	0	0	50

globulin from fraction III using I^{131} labelled globulin and the persistance of the reagins as judged by skin tests. The reagin test is not accurate but as far as could be judged the rate of disappearance of inert globulin and of reagin was the same, and there was nothing to suggest that reagins showed any exceptional ability to remain fixed in the skin It is believed that earlier reports to the contrary were due to the exceptional sensitivity of the test and the difficulties in making it quantitative.

Summary

Serum from allergic individuals has been fractionated on diethyl amino ethyl cellulose and it has been found that the reagins are largely confined to one fraction of γ_1-globulin.

References

1. *Kuhns, W J* J exp Med *99*, 577, 1954.
2. [illegible]

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[illegible], and *H Küstner* Zbl Bakt *80*, 160, 1921
9 *Pappenheimer, A M* J Immunol *75*, 259, 1955.

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The different fractions were assayed for reagin content by skin tests and the area of weal measured at different dilutions of the fractions, which had all been brought back to the original volume of the serum put on the column, by pressure dialysis. The results with one serum from a person sensitive to pollen are given in Table I. The euglobulin fraction is the small amount of globulin which comes out of solution on dialysis of the serum against 0.01 M sodium phosphate pH 7.5 prior to chromatography. It can be seen that the reagins are largely confined to fraction III and are absent from fraction I, the γ_2-globulin.

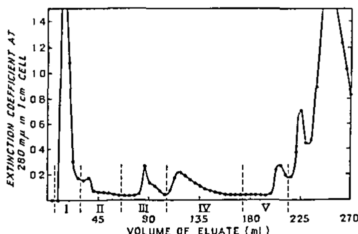


Fig 1 Chromatography of 3 ml human serum on 3 g diethyl amino ethyl cellulose (Column 12 × 1 1 cm) 0–45 ml eluting with 0 01 M sodium phosphate pH 7 5 45–180 ml eluting with 0 02 M sodium phosphate pH 6 2 180–270 ml eluting with 0 05 M sodium dihydrogen phosphate

Similar results were obtained with five other sera containing reagins against a variety of antigens. In some the reagins were entirely in fraction III and in others were more widespread, though always with the highest content in fraction III.

These results confirm those of others (1, 2) that the reagins are largely in the γ_1-fractions which contain only about 10% of the total γ-globulin of the serum.

Some properties of these purified reagins were investigated It was confirmed that they were labile to heating at 56° for 4 hours as has been reported using whole serum (9). The suggestion that reagins show exceptional persistence in the skin at the site of injection was also examined by comparing the persistance of the γ-

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Serum from allergic individuals has been fractionated on diethyl amino ethyl cellulose and it has been found that the reagins are largely confined to one fraction of γ_1-globulin.

References

1 *Kuhns, W J* J exp Med *99*, 577, 1954.
2 *Brattsen, I, H Colldahl* and *A H F Laurell*· Acta allerg, Kbh *8*, 1, 1955
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Schnyder, U W. Int Arch Allergy *11* 64–72 (1957)

The Importance of Intracutaneous Tests in Various Types of Constitutional Neurodermatitis

Dermatological Clinic of the University of Zurich, Switzerland
(Dir Prof *G Miescher*, M.D)

By U W SCHNYDER

Prior to the 1930's many physicians believed that an immediate skin reaction in response to intracutaneous injection of test materials proved that the patient with neurodermatitis was allergic to such materials – and that the allergenic substance in part or in whole was responsible for the patient's neurodermatitis. Today, many dermatologists feel that the immediate positive skin reaction does not necessarily indicate that the tested material has any etiologic relationship to the patient's neurodermatitis However, many reputable allergists believe that the immediate positive skin reactions to inhalant allergens in persons with neurodermatitis indicate a strong probable etiologic relationship to the neurodermatitic skin ailment.

Here, the term " constitutional neurodermatitis" is used as it has been by *Marchionini* and *Miescher*. Thus, it is the equivalent of *Besnier*'s "prurigo diathésique à forme objective eczémato-lichénienne". However, "constitutional neurodermatitis" is not the equivalent of atopic dermatitis as it has been described by *Sulzberger* and others. Atopic dermatitis includes specific skin manifestations occurring in infants, children and adults. The present author classifies the eczema of childhood, called "Fruhexsudatives Eczematoid" by *Rost* and *Marchionini*, as being different from the other forms.

The author will not discuss here whether or not the constitutional eczema of childhood (infantile phase of atopic dermatitis) has the same etiologic basis as the neurodermatitis of older children.

With increasing age, there is an increasing likelihood that a person with constitutional neurodermatitis will also develop asthma or allergic rhinitis, or both. Furthermore, it may take many years before potential allergies in children become manifest as they grow older and become adults.

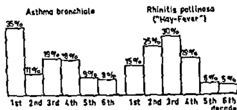


Fig 1. Date, measured in decades of years, of first appearance of allergies of the respiratory tract *(Bray)*

Although 33% of children with neurodermatitis will also have asthma, this correlation is found in more than 50% of the adult cases. In very small children, "constitutional dermatitis" and allergic rhinitis rarely coexist. However, in patients with neurodermatitis who are over 16 years of age, more than 40% also suffer from allergic rhinitis.

When do asthma and hay-fever occur for the first time in persons *not subject* to constitutional neurodermatitis? Fig. 1, based on the work of *Bray*, shows that 35% of persons developing asthma do so in the first *decennium* with relatively few persons suffering initial attacks at a more advanced age. However, only 15% of persons without skin manifestations develop the first

Only patients over the age of 16 were studied in this evaluation of the intracutaneous allergy tests in constitutional neurodermatitis. There were 136 neurodermatitis subjects over the age of 16. These patients were divided into two groups: a) those without respiratory allergies, and b) those with respiratory allergies. The group (b) was further subdivided into three groups: 1. neurodermatitis with

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However, for the entire group of 94 patients with neurodermatitis and allergies of the respiratory tract, 70 patients (75%) had positive tests. In general, persons with neurodermatitis combined with allergic rhinitis showed stronger positive skin reactions than those with neurodermatitis and asthma.

Sulzberger today regards skin tests positive to inhalant allergens as indicative of an allergic predisposition; while others (*Nexmand* in Denmark and *Tuft* in the United States) still regard the inhalant allergens as largely responsible for at least the seasonal worsening of the neurodermatitis.

	Winter	Summer	Autumn	Spring	None	Total cases
All cases	40%	18%	10%	11%	21%	131
Neurodermatitis with manifest allergies of the respiratory tract	34%	19%	8%	12%	27%	89
Neurodermatitis without manifest allergies of the respiratory tract	54%	15%	13%	8%	10%	42

Fig 3 Seasonal exacerbation of various types of neurodermatitis.

Fig. 3 indicates that 40% of the 131 cases of neurodermatitis showed worsening during the winter; 21% showed no seasonal variation; 18% showed worsening in the summer, 10% in the autumn, 11% in the spring. However, 34% of the patients with both neurodermatitis and respiratory allergies had exacerbation of their dermatologic symptoms in the winter; 27% had no seasonal exacerbation; 19% had worsening in the summer; 8% in the autumn, 11% in the spring And 54% of the patients who had neurodermatitis without respiratory allergies showed worsening

bronchial asthma; 2. neurodermatitis with allergic rhinitis; 3. neurodermatitis with both allergic rhinitis and asthma.

78 patients with neurodermatitis (57%) had positive skin tests, mainly to inhalant allergens; 58 patients (43%) had negative reactions. These findings agree with those of other authors. *Sulzberger* showed that 50% of his patients gave positive skin tests; *Schuppli*, 50%; *Alexander*, 52.7%, and *Blumenthal* and *Jaffé*, 50%.

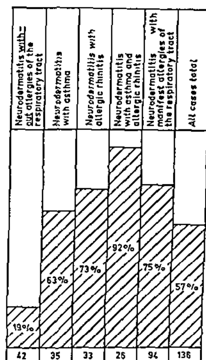


Fig. 2 Frequency of positive wheal-reactions in various types of constitutional neurodermatitis

Fig. 2 shows the frequency of positive wheal reactions in neurodermatitic patients with or without respiratory allergies. Only 19% of patients with neurodermatitis uncomplicated by respiratory allergies had immediate positive skin reactions to inhalant allergens. But in neurodermatitis complicated by asthma the percentage of positive skin tests was 63%; in neurodermatitis with allergic rhinitis, the percentage of positive skin tests was 73%; and in patients with neurodermatitis who had both asthma and allergic rhinitis, the percentage of positive skin tests was 93%.

However, for the entire group of 94 patients with neurodermatitis and allergies of the respiratory tract, 70 patients (75%) had positive tests. In general, persons with neurodermatitis combined with allergic rhinitis showed stronger positive skin reactions than those with neurodermatitis and asthma.

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in the winter of their skin ailment; 10% *showed no seasonal variation* in this; 15% had worsening in the summer, 13% in the autumn, and 8% in the spring. Thus, worsening of the neurodermatitis in the winter was more likely to occur in the group without respiratory allergies; whereas no seasonal pattern of exacerbation was more likely to occur in the neurodermatitic patients who had respiratory allergies. The incidence of exacerbation of skin symptoms during summer, spring and autumn was about the same in persons with neurodermatitis whether or not they had concomitant respiratory allergies.

	Winter	Summer	Autumn	Spring	None	Total cases
Seasonal dependency of positive skin-reactions among neurodermatitis cases	31%	16%	10%	14%	29%	74
Seasonal dependency of negative skin-reactions among neurodermatitis cases	54%	23%	9%	5%	9%	55

Fig. 4 Seasonal dependence of positive and negative skin-reactions among neurodermatitis cases

Fig. 4 shows the seasonal dependence divided according to neurodermatitis patients yielding positive skin-tests and those yielding negative skin-tests. In both groups those cases with more marked symptoms predominated in winter. In addition, the *frequency of seasonal dependence among those yielding positive* skin-tests is about three times that of the patients yielding negative skin-tests. Yet the frequency of cases worsening in summer, spring *and autumn is approximately the same in both groups.*

From this juxtaposition it may be seen that those subjects with positive skin-reactions and those without do not differ as regards seasonal dependence, but with respect to seasonal independence, those with positive skin-reactions show a clear superiority.

Nexmand and a number of other authors believe that allergies to pollen and to house-dust are to a large extent responsible for the seasonal exacerbations.

Fig. 5

Fig. 6

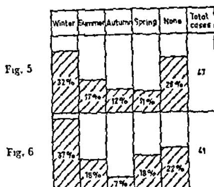


Fig 5 Seasonal dependence of neurodermatitis with hypersensitivity to pollen
Fig 6 Seasonal dependence of neurodermatitis with hypersensitivity to house-dust.

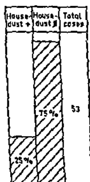


Fig. 7 *Neurodermatitis* patients with exacerbation in winter

Fig. 5 and 6 show that in neurodermatitic patients with either pollen or house-dust allergy, the patterns of exacerbation of the skin ailment were more likely to occur in winter. Thus, for pollen hypersensitivity, 28% showed no season exacerbation; 32% had

cerbation; 16% summer, 7% autumn and 18% spring. Fig. 7

shows that 25% of house-dust-positive patients with neurodermatitis show exacerbations of their skin ailment in winter, and that 75% of the patients who give negative tests have winter exacerbation of their neurodermatitis.

	Climatic dependency	Climatic non-dependency	Total cases
Skin-test positive	73%	27%	57
Skin-test negative	64%	36%	36

Fig. 8. Climatic dependence with respect to positive and negative reactions among neurodermatitis patients

This suggests that worsening of symptoms in winter cannot be ascribed to house-dust sensitivity.

However, persons with neurodermatitis, whether they have positive or negative skin reactions, do react to climatic changes.

Fig. 8 shows the relationship of neurodermatitis to climatic changes in 93 cases. 27% of those with positive skin reactions and 37% with negative skin reactions did not react favorably to climatic changes. Thus, almost 75% did react favorably to changes in climate.

If a patient with neurodermatitis does give a positive immediate skin reaction to an intracutaneous injection of a test substance,

this does not prove that this substance has contributed in part or in whole to the creation of the patient's neurodermatitis. However, in many instances, the individual may have a hypersensitivity of the respiratory tract to this material. Furthermore, inhalation of the allergenic pollen may cause an exacerbation of the allergy of the respiratory tract (allergic rhinitis or asthma) without altering in any way the condition of the patient's neurodermatitis.

Summary

1. For the purposes of this paper, only patients with neurodermatitis over the age of 16 were studied. About 20% of those who had no evidence of asthma or allergic rhinitis reacted with a positive immediate reaction to skin testing with inhalant allergens. But approximately 75% of all patients with neurodermatitis and asthma or allergic rhinitis – or both – had positive immediate reactions to skin testing with inhalant allergens.

2. In general, 40% of all patients with neurodermatitis tended to have exacerbations of their skin ailment in the winter, whether or not they had concomitant respiratory allergies – or had positive immediate skin reactions. 21% show no seasonal variation in the severity of their neurodermatitis; 18% show worsening in the summer; 10% in the autumn; 11% in the spring. A detailed analysis of the incidence of seasonal exacerbation of the neurodermatitis in both the allergic and non-allergic groups is given in the text and charts.

3. There was no difference between the subjects with positive or negative immediate skin reactions in the variation of the severity of the neurodermatitis with climatic conditions.

4. Patients who have constitutional neurodermatitis associated with allergic rhinitis generally give stronger positive skin reactions to the test materials than those patients with constitutional neurodermatitis and asthma.

5 There is no proof that an immediate positive skin test to inhalant allergens indicates that the specific inhalant material is in part or in whole responsible for the neurodermatitis.

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Nickel Sensitivity in Women

By C. D CALNAN

Consultant Dermatologist, St. John's Hospital for Diseases of the Skin, London

It has been known for many years that the skin can become sensitized to nickel. Most investigators, whether dermatologists or allergists, who have studied patients with contact dermatitis or eczema (I shall use these terms synonymously), quickly appreciated the high incidence of positive reactions to nickel. More than four years ago Dr. *Meara* and I started a special clinic at St. John's Hospital for Diseases of the Skin, for patch testing cases of allergic dermatitis. We do not perform routine or "battery" patch tests in cases of eczema, and the patients referred to our clinic are selected by the physician as cases of possible contact dermatitis which may be of the allergic rather than the irritant or toxic type.

The number of patients tested, those with positive tests, and the nickel sensitive are shown in Table I.

Table I
Patch Test Clinic Results

	Patients Tested	*Total Positives*	*Nickel Positives*
1953	1028	478	131
1954	891	412	198
1955	885	420	180
1956	931	489	146

Nickel is quite obviously the commonest cause of allergic contact dermatitis in this hospital. Almost all the cases are women, and very few work in industrial nickel processes such as refining or plating. In fact, the sensitizing contact agents are stocking suspenders, earrings, or other clips and fasteners which touch the

skin. The high incidence of this non-industrial nickel sensitivity led us first to make a detailed clinical study, and then to carry out certain research investigations.

Clinical Features

The clinical picture which these patients present is often rather complex, in that they show areas of dermatitis, related and unrelated to metal contact, and the clinical course is very variable. An analysis of four hundred cases (*Calnan*, 1956) has enabled me to classify their lesions into three groups:

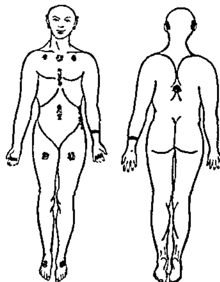
1. Primary – areas in direct contact with metal;

2. Secondary – selective symmetrical areas which are involved when the dermatitis spreads;

3. Associated – areas of dermatitis which appear to have no relationship to the nickel sensitivity.

Fig 1. Nickel contacts

Suspenders
Brassiere
Earring
Necklace
Zip fasteners
Spectacles
Brooches
Corsets
Scissors, etc.
Pins
Hair grips
Watches
Bracelets
Shoe buckles



The primary sites are shown and listed in fig. 1. This list is not, of course, comprehensive and, in fact, any area of the skin may become affected if it is in contact with nickel. Table II gives the sites first affected in 400 patients, and it will be seen that stocking suspenders account for 95%.

Table II
Initial Cause of Nickel Dermatitis

Suspenders	381
Earrings	13
Wrist watch	3
Brassiere clips	1
Necklace clasp	1
Spectacle frame	1

Once the patient is sensitized to nickel, it is very noticeable that she does not react to every contact with it. Only one, two or three suspender sites may be affected, while earrings, brassiere clips, or a watch buckle may produce no reaction. And sometimes the patient may wear suspenders and other nickel plated fasteners with impunity. This aspect of the problem will be mentioned again later.

The secondary sites (fig. 2) which are quite specific, are as follows:

1. Elbow flexures
2. Eyelids
3. Sides of neck and face
4. Inner thighs
5. Generalised.

This secondary eruption occurs at some time in 75% of cases. It appears to be a very special type of spread which does not occur in any other variety of contact dermatitis, and, as far as I know, does not occur in the nickel dermatitis encountered by workers in refineries or in nickel plating works. It is in fact so frequent and so characteristic that one can recognise cases of nickel sensitivity from this eruption alone. Very rarely, the secondary eruption may take another form such as erythema multiforme, urticaria, generalised pruritus or prurigo. An associated eczema is seen in a third of the cases. In some instances it is of a pattern which would appear to be fortuitous and coincidental, for example, varicose, seborrhoeic, nummular or atopic eczema. But in the majority it is an eczema of the hands, which is present in a fifth of all cases (Table III). I am quite unable to attribute the hand eczema to direct nickel contact, as is sometimes suggested (*Waldbott*, 1953). *Kroepfli* and *Schuppli* (1955) found nickel to be present in small amount in household detergents, but we have examined a number

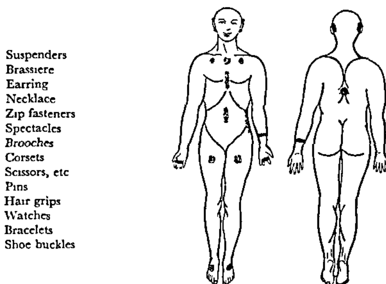
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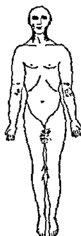
Fig. 1. Nickel contacts



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but neither of them met with success. But such a process is produced quite frequently by certain infective agents, such as fungi and tubercle bacilli, and is known as the id phenomenon. There is a primary lesion at the site of the infective agent, and a secondary eruption produced by haematogenous dissemination of this agent

Fig 2
Secondary Spread



or some product of it. This is accompanied by allergic hypersensitivity to an antigen obtained from the agent. Most typically this id phenomenon is shown with an acute trichophyton infection of the foot, associated with a symmetrical vesicular id eruption on the palms and fingers, and a positive trichophytin skin test. The lesions on the hands are presumably produced via the blood stream by trichophytin from the foot lesion.

Nickel dermatitis in women would seem to be very similar. A primary eruption presents on the thighs at the site of nickel contact. A secondary, symmetrical eruption appears on the elbow flexures, eyelids, and so on; and allergic hypersensitivity to the primary causal agent is demonstrated by a positive patch test to nickel sulphate. The secondary eruption must be chemically mediated in some way from the primary site. As far as we know, the antibodies of contact dermatitis are present in the lymphocytes and plasma cells and are not circulating freely in the serum. Nickel sensitivity dermatitis of the type described above is unique in produc-

Table III

Cases with Associated Eczema

Eczema of hands	81 = 20%
Seborrhoeic	13
Exogenous	12
Unclassified	10
Atopic	4
Nummular	3
Hypostatic	1
	124 = 31%

of such products available in this country with negative results (*Wells*, 1956).

One may digress here to consider briefly the spread of eczema. For a tendency to spread and recurrence are special features of eczema. It spreads in three ways:

a) by direct extension;

b) by contralateral extension – e.g. from one foot or leg to the opposite foot or leg;

c) by eruption of lesions at a distance – so called autosensitization.

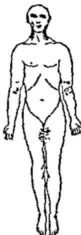
The initial site has some influence on the spread. Any eczema on the lower leg has a strong tendency to disseminate widely. *Haxthausen* (1955) found this type of spread in 37% of 235 cases of leg eczema. But it occurs in 75% of cases of suspender dermatitis, and in these the pattern is much more distinct and localised than that of the spread from lower leg eczema. Suspender dermatitis is caused by a known allergen, while the leg eczema is not due to a specific allergen. Hence it should be possible to study the mechanism of this spread.

Whitfield (1921) used the term autosensitization or autogenous spread for this phenomenon. He postulated that the skin became sensitized to some agent circulating in the serum to explain the appearance of eczema produced by the fluid from bullae spreading on to normal skin, and suggested that this agent could circulate by the blood stream and produce lesions elsewhere.

There is, however, no absolute proof to support this view, in spite of much suggestive experimental work (*Lowenthal*, 1954). Attempts have been made by *Dowling* (personal communication) and *Wells* (personal communication) to spread eczema by applying serum from eczematous vesicles and bullae to areas of normal skin,

but neither of them met with success. But such a process is produced quite frequently by certain infective agents, such as fungi and tubercle bacilli, and is known as the id phenomenon. There is a primary lesion at the site of the infective agent, and a secondary eruption produced by haematogenous dissemination of this agent

Fig 2
Secondary Spread



(Elbow flexures
Forearms)
Eyelids
Neck and face
Inner thigh
Generalised

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ing an id eruption, for no other contact allergen produces it. So far we have been unable to detect any agent in the serum to mediate it. Passive transfer tests are negative. But the reaction is a very sensitive one, for patch tests may easily flare it up, and patients show recurrences at the secondary sites much more readily than at the primary sites. Although many other eruptions are called eczematides and seborrhoeides, in none is there any proof of allergic hypersensitivity.

If nickel is given by mouth to a sensitive patient no reaction occurs (*Wilson*, 1956, personal communication). It is known that contact sensitizers vary greatly in this respect; for instance sulphonamides and penicillin often elicit a reaction, while rhus, amethocaine, and many others rarely do so.

Patch Tests

Positive patch tests are elicited with a 2½% solution of nickel sulphate. Many patients will react to weaker concentrations, and exceptionally a 5% solution is required. Nickel-containing coins can be substituted. The appearances of positive reactions are variable, and pustular lesions are not uncommon. Cross sensitivities to cobalt do occur in about half the cases (17 out of 32). A number of patients show positive reactions to other substances (Table IV). In most cases these are unrelated. Nickel sensitivity has become so frequent that one sees patients who have a dermatitis from lipstick, rubber, or a proprietary ointment, and who give a history of "metal rash" in addition.

Table IV
Associated Sensitivities

Rubber gloves	Streptomycin
Primula	Antihistamine
Lipstick	Shoes
Hair Dye	Face powder, lipstick, stockings
Quinine	Paraphenylenediamine
Anthisan	Hair net elastic
Benadryl	Rubber

Experimental

There are a number of special features in nickel suspender dermatitis which need some explanation. Patients who are found

to be nickel positive are often able to wear the metal in contact with the skin but show no reaction. Their degree of sensitivity has not apparently varied; hence some other factors must play a part in deciding whether sensitive skin will react to metal contact. Sweat, friction and penetration are important. If a nickel plated stocking suspender or a clip or a nickel coin is placed in a solution of artificial human sweat for 12 hours, it can be shown that nickel has been dissolved off from the metal. It can be detected by a spot test with dithio-oxamide or dimethyl glyoxime (*Feigl*, 1949). The former is more suitable for tissues but not so specific as it also reacts with copper.

Wells (1956) has shown that nickel has a peculiar affinity for keratin He immersed sections of skin in a solution of nickel sulphate and then stained them with one of the two agents mentioned above for the spot test. Nickel was seen to be located especially in the deeper layers of the stratum corneum and the inner root sheath of hairs. Keratin will take this up from a solution of nickel sulphate at a dilution of one part per million. But the nickel keratin link is a very weak one as it is readily removed by washing or by a change in pH to the acid side. From the use of certain histochemical methods, *Wells* thought it probable that the nickel was joined by the carboxyl groups of keratin.

In an attempt to study this linkage further, *Magnus* (unpublished) has used electrophoresis with kerateine. This is a soluble protein, in which the disulphide bonds of keratin are broken with potassium cyanide. Kerateine and nickel sulphate are mixed in equal parts at various pH levels. When an electrophoretic current is passed the nickel moves with the kerateine if it is linked, but separately if not linked. Nickel passes rapidly towards the cathode, and the kerateine more slowly in the direction of the anode. The results show that at pH 2–5 there is no linking, while at pH 8–11 the metal appears to be entirely linked, because both move together. At any intermediary pH 6–8 there is evidence of partial linkage. The paper is stained with brom-phenol-blue for the protein, and with rubianic acid for nickel.

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comr [illegible] to nickel is so

[illegible] manifestations so unique that I feel it offers great opportunities for the study of allergic dermatitis.

Summary

Non-industrial nickel sensitivity in women caused by stocking suspenders is now very common in England. The clinical pattern of dermatitis is quite distinctive. It consists of primary lesions at sites of direct contact, a secondary symmetrical eruption of the "sensitization" type, and patches of associated eczema which do not appear to be directly related to the nickel sensitivity. A fifth of all cases have an eczema of the hands.

Evidence is presented that the secondary eruption is a true allergic eczematide. The high incidence of nickel dermatitis may be attributable to the strong affinity of keratin for nickel, which has been shown experimentally.

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Histochemical Investigations and Classification of the Reactive Dermatoses in Japan

By KENTARO HIGUCHI (Fukuoko)

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Experimental Studies on the Pathogenesis of Contact Eczema in the Guinea-Pig

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I Introduction

In spite of the difficulties in defining and classifying the clinical conditions belonging to the group called eczema, everyone will agree with the meaning of the word "contact eczema", "contact-dermatitis" or "contact type eczematous dermatitis", a condition frequent in man and easily produced in laboratory animals. Besides its clinical and biological importance, contact eczema is of special interest because its pathogenic mechanism is today the best working hypothesis towards studying the other more complete forms of "eczematic diseases". Although important findings show that the pathogenic mechanism of the contact eczema may be an allergic one, this mechanism is today not yet completely understood. Questions as important as the fate of the antigen after contact with the skin, the place where antibodies are produced and the way by which the whole skin becomes sensitized are still unknown.

The purpose of our study of contact eczema was therefore to analyse its different steps, from the first contact with the sensitizing agent until the appearance of the generalized sensitization, in order to determine the systems or organs involved in the process and to establish the time in which the various phenomena occur.

II. Basic Experiment

White *guinea-pigs* weighing 500 g are used (fig 1) Th

Summary

Non-industrial nickel sensitivity in women caused by stocking suspenders is now very common in England. The clinical pattern of dermatitis is quite distinctive. It consists of primary lesions at sites of direct contact, a secondary symmetrical eruption of the "sensitization" type, and patches of associated eczema which do not appear to be directly related to the nickel sensitivity. A fifth of all cases have an eczema of the hands.

Evidence is presented that the secondary eruption is a true allergic eczematide. The high incidence of nickel dermatitis may be attributable to the strong affinity of keratin for nickel, which has been shown experimentally.

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test) the following factors, which are under our control, may play an important rôle:

a) concentration of the solution

b) volume of the solution applied

c) area of skin to which the solution is applied.

As we work only with DNCB dissolved in acetone, we do not consider here the rôle played by the nature of the antigen and the nature of the solvent a question perfectly analysed in the paper of *de Weck* and *Brun*. As acetone will evaporate rapidly, we obtain a certain amount of DNCB distributed uniformly on a certain area of skin.

In order to work always under identical and reproducible conditions the following technique was developped:

a) With a metallic print, circular areas are marked on the shaved flanks of the animals.

b) With a micropipette, exactly measured volumina are applied as uniformly as possible to the marked area.

c) Reactions are read 24 hours later.

d) The degree of the reaction is evaluated according to a scale.

The skin areas used, the volumina applied, the amounts of DNCB per cm² and the scale used to evaluate reactions are indicated in table I.

2. *Toxic threshold*. To determine the toxic threshold, several groups of guinea-pigs, which had never had previous contact with

Table I

Quantitative Epicutaneous Test

Circular test-area cm²	Volume applied cm³
2	0,025
4	0,050
8	0,100

Degree of reaction	Macroscopic alterations
0,5	Isolated red spots
1	Diffuse slight redness
2	Strong redness and slight swelling
3	Deep redness and strong swelling

Concentration of the test solution ‰ DNCB	DNCB per skin-area µg / cm²	
0,3	3,75	not primary toxic
0,5	6,25	
0,9	11,25	
1	12,50	primary toxic
1,5	18,75	
2	25,00	
3	37,50	

is used, which is applied directly upon the skin. The first application performed in order to sensitize the animals is called *primary contact* and the place where it is done *contact place*. Second or further applications performed to find out if animals became sensitized are called *test*. The time elapsed between primary contact and test is called *incubation time*. Once sensitized, the animals conserve this property for a long time, perhaps permanently.

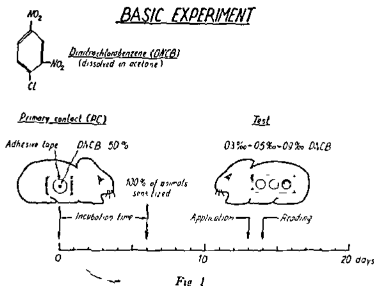


Fig 1

At high concentration DNCB may damage the skin of every normal animal. The lesion so produced is called *primary toxic lesion*. The lesion produced in sensitized animals by DNCB in concentrations innocuous to normal animals is called *allergic reaction* (= test). A weak primary toxic reaction cannot be differentiated macroscopically from a strong allergic one. If macroscopic observation of skin reactions is used in order to recognize whether an animal has become sensitized, this can be obtained only by applying a *test dose certainly not primary toxic*. Therefore, before any other study can be done, a quantitatively exact *testing technique* has to be developed with which to establish the primary toxic and the allergic threshold.

III. Toxic and Allergic Threshold

1. *Technique of the epicutaneous test.* When testing skin sensitivity by application of a solution of antigen on its surface (epicutaneous

arily non-toxic solutions of DNCB. The number of animals, skin areas, concentrations used and the results obtained are indicated in table IV. They show that with increasing strength of primary contact the percentage of high sensitized animals becomes more and more frequent. Plate I, fig. 2 shows the macroscopic aspect

Table IV
Determination of the Allergic Threshold Concentration

Technic of sensitization	Concentration of antigen in ‰	Positive reactions / Number of animals	Positive reactions in %
39 animals were sensitized by daily application of *primarily non-toxic* doses of DNCB in acetone. Tests performed later than 10 days after the primary contact	0,3 0,5 0,9	31 / 187 79 / 187 149 / 187	16,6 42,2 79,7
16 animals sensitized by a single application of 0,05 cm³ of a *1%-solution* of DNCB in acetone on a skin-area of 4 cm². Tests 9-17 days after PC	0,3 0,9 0,9	20 / 88 76 / 88 76 / 88	22,7 86,4 86,4
14 animals sensitized by application of some *DNCB-crystals* on an explantate with vascular pedicle and skin bridge. Tests 76-91 days after PC	0,3 0,5 0,9	45 / 84 70 / 84 80 / 84	53,6 83,4 95,3
26 animals sensitized by a single application of 0,002 cm³ of a *50% DNCB-solution* in acetone on a circular skin area of about 3 mm diameter subsequently covered by a small adhesive tape. Tests later than 14 and more days after the PC	0,3 0,5 0,9	64 / 84 76 / 84 84 / 84	76,2 90,5 100,0

of allergic reactions. A clear difference in their intensity can be observed corresponding to the different strength of the testing solutions applied. The results of the toxic and allergic threshold expressed as curves on fig. 2 show that comparing virginal with high sensitized animals there is no overlapping of values.

Conclusion: Solutions of 0,9-0,5 and 0,3‰ are appropriate for testing sensitized animals.

DNCB *(virginal animals)* received an application of dissolved DNCB at different strength on two consecutive days. Concentrations used and results obtained are indicated in table II. They show that even when solutions of 1‰ are applied, 12,5% of the animals

Table II

Determination of the primary toxic threshold concentration of DNCB in acetone on the guinea-pig by application of 0,05 cm³ on a skin-area of 4 cm²

Experiments	Number of animals	Number of tests per concentr	Test-concentration 1‰ Animal pos		1,5‰ An pos %		2‰ An pos %		3‰ An pos %	
a	8	16	2	12,5	14	87,5	16	100	16	100
b	8	16	1	6,2	9	56,2	14	87,5	15	93,8
c	8	16	2	12,5	10	62,5	12	75,0	16	100
d	8	16	2	12,5	12	75,0	14	87,5	16	100
e	8	16	5	31,2	2	12,5	13	81,3	16	100
f	8	16	0	0	1	6,2	12	75,0	14	87,5
Total	48	96	12	12,5	48	50,0	81	84,4	93	97,0

develop primary toxic reactions. Applying solutions of DNCB in acetone of 0,9–0,5 and 0,3 ‰ – which were currently used to test sensitized animals – to 149 virginal animals a weak reaction to 0,9 appeared in only one of them (table III). The macroscopic aspect of primary toxic reactions, as we shall see later, cannot be distinguished from the allergic one (plate I, figures 1 and 2).

Table III

Behaviour of non-sensitized normal animals against the acetonic solutions of DNCB used for the testing of allergic animals. Volume applied 0,025 cm³, area 2 cm²

Concentration of the applied solutions ‰ DNCB	μg DNCB	positive reactions	total of the tested animals
0,3	3,75	0	149
0,5	6,25	0	149
0,9	11,25	1	149

Conclusion: Appropriate concentrations to test sensitized animals may be weaker than 1‰. Positive tests obtained with higher concentrations may be due partly to toxic reactions.

3. *Allergic threshold.* Groups of guinea-pigs sensitized by primary contacts of increasing intensity were tested several times with prim-

Table V
Determination of the Incubation Time

Animals sensitized with	Concentration	Number of animals	Number of positive animals after days 4	5	6	7	8	9	10	11	12
Primarily non-toxic doses	0,50‰	21	0	0	0	14	6	1	0	0	
	0,75‰	34	0	0	20	4	8	2	0	0	
	1,00‰	26	0	0	4	10	9	2	0	1	
	1,00‰	8	0	0	1	4	2	1	0	0	
	1,00‰	8	0	0	0	2	5	1	0	0	
	Total	97	0	0	25	34	30	7	0	1	
Positive animals in percent of the total			0	0	25,8	35,0	31,0	7,2	0	1,0	
Sum of the percents			0	0	25,8	60,8	91,8	99,0	0	100,0	
Primarily toxic doses	50%	24	0	16	23						24
	50%	8		6	8						
	50%	8			8						
	50%	8				8					
	50%	8					8				
	50%	8						7			8
	Total	64	0	22	39	8	8	7			32
Number of tested animals			24	32	40	8	8	8			32
Percentage reacting positively			0	68,7	97,5	100,0	100,0	87,5			100,0



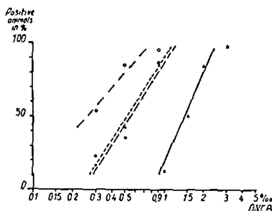


Fig 2 Allergic and primary toxic threshold of the guinea-pig to DNCB dissolved in acetone Dotted curves Animals with low sensitization Dash-point curves Animals with high sensitization Unbroken curve primary toxic reactions Abscissa logarithmically subdivided For numerical values see also tables II and IV.

IV. Incubation Time

The determination of the time clapsed between the primary contact and the appearance of the sensitization of the whole skin is important for different reasons. It may be necessary to know:

a) The earliest time when animals can be tested.

b) After how many days the highest percentage of animals may be positive.

c) At what time processes as important as the formation of antibodies take place, i.e. when corresponding alterations may be searched for in certain as yet unknown organs.

1 *Experiment.* Several groups of virginal animals received a primary contact of varying intensity Using primarily non-toxic doses, applications were done daily until the appearance of positive reactions. Using a 50% solution only one test was performed in each group at increasing time intervals after the primary contact.

2. *Results* reported in table V show that the incubation time is shorter in animals sensitized with high doses of antigen than in those which received primarily non-toxic doses. In any case no sensitized animals were found before 4 days after the primary contact. Between 5 and 9 days (after the primary contact) practically 100% of the animals had become sensitized. These results are again evident in the curves of fig. 3 where it is shown that strongly sensitized animals become positive practically two days earlier than those contacted with primarily non-toxic doses.

Table V
Determination of the Incubation Time

Animals sensitized with	Concentration	Number of animals	Number of positive animals after days 4	5	6	7	8	9	10	11	12
Primarily non-toxic doses	0,50‰	21	0	0	0	14	6	1	0	0	
	0,75‰	34	0	0	20	4	8	2	0	0	
	1,00‰	26	0	0	4	10	9	2	0	1	
	1,00‰	8	0	0	1	4	2	1	0	0	
	1,00‰	8	0	0	0	2	5	1	0	0	
Total		97	0	0	25	34	30	7	0	1	
Positive animals in per-cent of the total			0	0	25,8	35,0	31,0	7,2	0	1,0	
Sum of the percents			0	0	25,8	60,8	91,8	99,0	0	100,0	
Primarily toxic doses	50%	24	0	16	23						24
	50%	8		6	8						
	50%	8			8						
	50%	8				8					
	50%	8					8				
	50%	8						7			8
Total		64	0	22	39	8	8	7			32
Number of tested animals			24	32	40	8	8	8			32
Percentage reacting positively			0	68,7	97,5	100,0	100,0	87,5			100 0

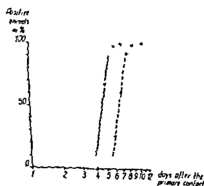


Fig 3 Incubation time of experimental contact eczema in the guinea-pig after primary contacts of different strength Dotted line: Using primarily non-toxic solutions (0,5–1‰) Unbroken line. Using primarily toxic solution (50%) Abscissa logarithmically subdivided For numerical values see table V.

V. Minimal Contact Time

Dealing with the question: what tissues or organs are involved in the process of sensitization, it may be of importance to know:

a) How long do the pathogenetic events remain localized to the contact area.

b) By how long can the development of generalized sensitization be avoided for instance by surgical excision of the place of primary contact.

1. *Experiment.* In several groups of animals the contact place on the skin was freely excised at increasing intervals after the primary contact. For each interval a correspondent control-group of animals received a primary contact but without later removing the contact place. All the animals were tested 10–14 days after the primary contact.

2. *Results.* Table VI shows that no positive animals appeared when the contact place is removed before 12 hours. If the contact place remains 16 hours, 56% of animals become positive, 77% if it remains 24 hours and 100% if it remains 32 hours. For technical reasons, we consider the "100% Minimal Contact Time" to be 48 hours. These results are again evident on the curve of fig. 4 based only on groups of more than 10 animals.

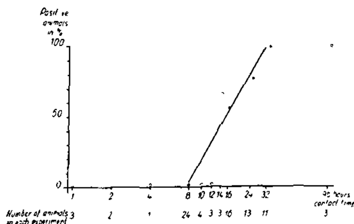


Fig 4 Minimal contact time after primary contact with 20–50% DNCB in acetone • Points based on more than 10 animals o Points based on 4 or less animals Abscissa logarithmically subdivided For numerical values see table VI

3. *Conclusions.*

a) Compared to the incubation time the Minimal Contact Time is relatively short.

Table VI

Minimal Contact Time

Minimal contact time of the DNCB applied to the skin necessary for the development of the sensitization, determined by excision of the contact place at increasing time intervals

Contact place excised after the following hours	1		2		4		8		10		12		14		16		24		32		96		Control animals without excision of the contact place	
Results after 10-12 days	Number of animals																							
Experiments	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—
a	0	3															2	0			3	0	3	0
b			1*	1	0	1	0	4							2	0							2	0
c							0	4							3	0								
d									0	4	0	3			0	4								
e															1	3								
f													2	1	3	0								
g							0	4									2	3					4	0
h							0	6									6	0					6	0
i							0	6											6	0			5	0
k																			5	0			6	0
Total positive animals in %	0	3	1*	1	0	1	0	24	0	4	0	3	2	1	9	7	10	3	11	0	3	0	26	0
	0		50*		0		0		0		0		66		56		77		100		100		100	

* primarily toxic reaction

b) Pathogenetic events remain localized to the contact place for only a short time.

c) Speaking in terms of time the greater part of the sensitizing process seems to occur in organs other than the skin.

VI. Pathways by which Influences Emerging from the Contact Place are Transmitted

The previous experiment indicates that the skin seems not to be the only organ involved in the sensitization process. Influences emerging from the contact place may reach other organs where, let us assume, antibody-like factors are formed which may reach the skin causing its sensitivity. Theoretically, these influences may be transmitted by the following ways:

a) Blood path
b) Lymphatic system
c) Nervous system
d) Skin considered as a syncytium.

To resolve this problem, each system had to be examined, separately from this point of view. This was partially possible by the following technique.

1. *Experiments.* Skin fragments were isolated from the surrounding and underlying tissues but kept in connection with the organism by the *vascular pedicle* normally irrigating this fragment. By this way the skin fragments – we call them *explantates* – were kept alive for two to four weeks. This is corroborated because the explantates are sensitive, blood appears when they are cut with scissors and especially by the fact that hair goes on growing as on the normal skin.

Operative technique: The animals are anesthetized with ether with an apparatus specially developed (fig. 5). A round island of skin is cut by scissors and separated from the surrounding and underlying tissue (plate I, figures 3–8, plate II, figures 1–3) At the same time the arterio-venous pedicle normally irrigating this skin area is prepared. Afterwards the wound is closed with Michel-clamps but permitting the pass of the arterial pedicle The explantate is then extended over a gauze and fixed with adhesive tape, the whole covered again with gauze and fixed again with adhesive tape. Primary contact is done by applying to the explantate some

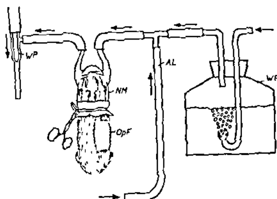


Fig 5 Apparatus for anesthesia (schematically) WP = Water pump NM = Mask for anesthesia OpF = Operation field AL = Rubber tube for air WF = Bottle with ether Arrows indicating the direction of current into the system

crystals of DNCB and more recently by applying 0,002 ml of a 50% solution of DNCB in acetone. The area of the primary contact is then covered with a little adhesive tape to avoid contamination of other regions of the skin. This operation is represented schematically in fig 9a.

At histological examination (plate IV, figures 1–2) the pedicle is composed of a great vein and some arteries and nerves surrounded by granular tissue.

2 *Results.* Several groups of animals treated this way were sensitized and tested at different time intervals as shown in fig. 6. In none of the 44 animals in which DNCB was applied to a skin fragment connected with the organism only by some arteries, veins and nerves, did sensitization appear. Using explantates prepared 5, 13 and 19 days before, it could be demonstrated that the absence of sensitization was not due to a state of surgical shock, in which these explantates could have been during the first post-operation days

3 *Conclusions* On the basis of these results we may postulate that influences emerging from the contact place are not transmitted by the pathways connecting the explantate to the organism i e, the blood and the nerve path and that this transmission will probably be done either by the lymphatic system or the skin.

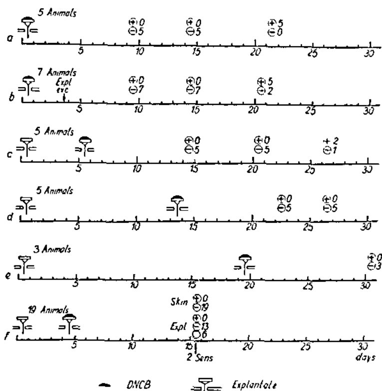


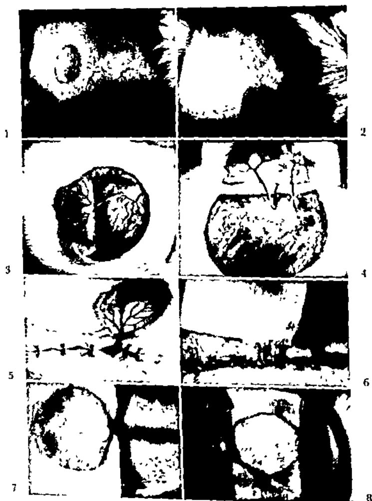
Fig. 6. Animals sensitized by application of the primary contact on a skin fragment – explantate – connected with the organism by a pedicle containing arteries, veins and nerves. Abscissa: days after beginning of the experiment. + — Test positive resp. negative. O Readings unobtainable.

VII. By which Pathways Do the Factors Responsible for Sensitization Become Generalized to the Whole Skin

1. *Experiment.* After the results obtained in our previous experiments it was extremely tempting to use the inversed disposition, i. e. to perform the primary contact on the normal skin and the test on the explantate as well as on the normal skin. This is represented schematically in fig. 9b.

2. *Results.* As shown in fig. 7, in 25 animals an explantate was prepared and immediately the DNCB applied to the normal skin. 8 to 14 days later the explantate and the normal skin were tested. The reactions on the explantate were positive in 15, negative in 5 and readings unobtainable in 5 cases. On the normal skin the reactions were positive in 21 and negative in 4 cases. The macroscopic aspect of a positive test on the explantate is shown on plate II, figures 4–5.

Plate I



Figs 1–2 Toxic and allergic reactions of different degree

Figs 3–8 Preparation of an explantate with vascular pedicle.

Plate II

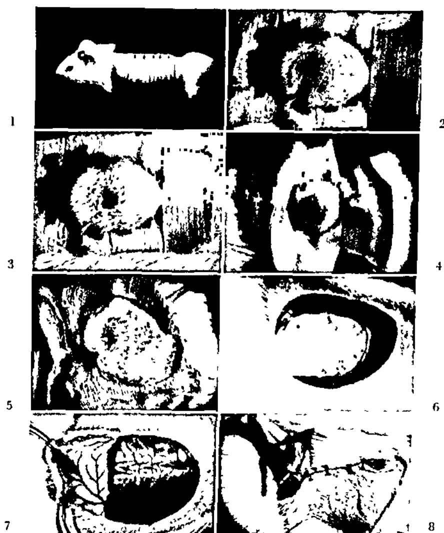


Fig. 1 Bandage with elastic adhesive tape

Figs 2–3 Primary contact on the explantate

Figs 4–5 Positive test on the explantate

Figs 6–8 Preparation of an explantate with vascular pedicle and skin-bridge

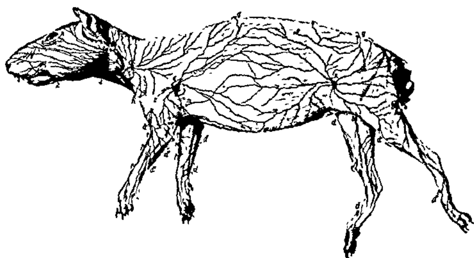
Plate III



1



2



3

Fig 1 Primary contact on an explantate with vascular pedicle and skin-bridge

Fig 2 Explantate with skin-bridge Incision to remove the subiliacal lymph nodes

Fig 3 Lymphatic vessels of the skin of the guinea-pig Lateral view with representation of the cutaneous muscles L = Lymphatic vessel, Ln = Lymphonodulus, Lc = Lymphocentrum 1 = Ln buccalis, 2 = Lc Submentale, 3 = Ln mandibularis; 4 = Lc parotideum, 5 = Ln cervicalis superficialis (cranialis), 6 = Ln axillaris externus s accessorius (caudalis), 7 = Lc subiliacum, 8 = Ln popliteus a = L to the Ln axillaris proprius (cranialis), b = L to the Ln femoralis medialis, c = L towards the depth to the Ln renalis, d = L turning to the lateral side and appearing again on d', e = L turning to the medial side of the thigh, f = L for the Ln popliteus, g = L crossing the median line to the opposite flank, h = L of the anterior and inferior region of the chest

Gerhard Keller Die Lymphgefäße der Haut des Meerschweinchens Zeitschrift fur Infektions-Krankheiten der Haustiere *52-53* 250 (1937-38)

Plate IV



Histological picture of a transverse section through the vascular pedicle *Fig 1* Section close to the entrance of the pedicle to the explantate Enlargement: ×80, *van Gieson* *Fig 2* Vein, arteria and nerves in the middle part of the pedicle Enlargement ×200, *van Gieson* V = strongly dilated vein; A = arteria partially thrombotic and recanalized, N = nerve, G = granulomatous tissue

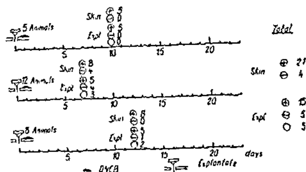


Fig. 7 Animals sensitized by application of the primary contact on the normal skin. Test performed on the explantate and the normal skin. Abscissa: days after beginning of the experiment. + — = Test positive resp. negative. O = Readings unobtainable.

3. *Conclusion.* Factors causing generalized sensitization are spread by the pathways present in the pedicle of the explantate, most probably by the blood path. In these experiments however the function of the nerves also present cannot be excluded.

VIII. *Significance of Lymphatic System in the Pathogenesis of Contact Eczema*

After these results it was justified to postulate that the influences emerging from the contact place would be transmitted to the organism either by the lymphatic vessels or the skin, as they were not conducted by the blood path or the nerves.

1. *Experiment* To resolve this question the already described explantate was so modified that beside the vascular pedicle the fragment was connected with the organism in addition by a *skin bridge.* The latter was orientated in such a way that it contained the afferent lymph vessels of the skin fragment to the inguinal lymph nodes The operative technique is shown in the plate II, figures 6–8 and plate III, figures 1–3. Two experiments were then performed, represented schematically in fig. 9c and d. In the first group of animals the primary contact was applied to the explantate and the test 15 days later on the normal skin. In the second group the regional lymph nodes were removed surgically just before the application of the DNCB on the explantate (primary contact) The primary contact and the test were then performed in the same way.

2. *Results.* As shown in the fig. 8 from the 32 animals which received their primary contact having their lymph nodes left in

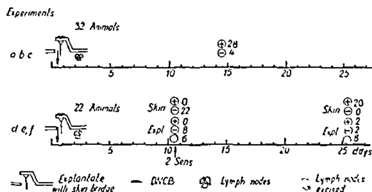


Fig. 8. Animals sensitized by application of the primary contact on an explantate connected with the organism by a vasculo-nervous pedicle and in addition by a skin bridge. Significance of the regional lymph nodes. Experiments a, b, c: lymph nodes left in situ. Experiments d, e, f: lymph nodes excised. Abscissa: days after beginning of the experiment. + — Test positive resp. negative O = Readings unobtainable.

situ, 28 developped positive reactions. On the other hand in none of the 22 animals in which the lymph nodes had been removed before the primary contact did sensitization appear. To show that these animals were not essentially refractory to sensitization, they were again sensitized, this time on the normal skin and all became positive.

Conclusions. The regional lymph nodes of the explantate are indispensable for the development of the contact eczema. By this experiment centripetal transmission via the nerves can be definitely excluded. Otherwise the animals of this group had become positive as the nerves were present in the pedicle.

IX. Conclusions

From these experiments we may postulate the following conception of the pathogenesis of contact eczema (fig. 9).

a) The influences emerging from the place of primary contact with the antigen (DNCB) are transmitted by the lymph vessels to the regional lymph nodes. In this function the nervous system has been excluded.

b) The regional lymph nodes are indispensable for the development of contact eczema, as demonstrated by their extirpation.

c) The regional lymph nodes may be only a part of the systems which transmit the influences emerging from the contact place to the organs where antibody-like factors are produced or may

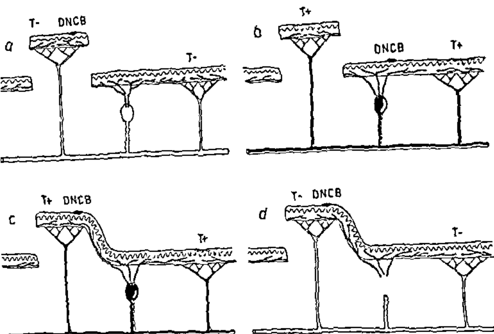


Fig 9 Experiments on cutaneous fragments (explantates) schematically represented The

[illegible]

and b) explantates with vasculo-nervous pedicle. c) and d): explantates with vasculo-nervous pedicle and skin bridge, in d) the regional lymph nodes have been excised

a) Primary contact performed on the explantate Lymphatic system not involved test on the [illegible]

c) Primary contact on the explantate Lymph nodes left in situ, Lymphatic system involved Generalisation by the blood path Test on the explantate and the normal skin positive

d) Primary contact on the explantate Lymph nodes previously excised Test on the explantate and normal skin positive Transmission of the influences emerging from the contact place via nerves contained in the pedicle is definitely excluded.

themselves produce such factors. The latter postulation is partially confirmed by *Skog* who obtained positive transfer experiments using among other agents lymph fluid from the thoracic duct.

d) We do not know whether beside the regional lymph nodes other organs or systems also participate in the production of antibody-like factors. These processes may be looked for especially during the incubation period, *i.e.* during the first 5 days after the primary contact.

e) We consider that the factors responsible for the sensitization of the entire skin are spread by the blood path. It seems less probable that transmission occurs via the nervous system.

We believe that this study contributed certain links clarifying the pathogenesis of contact eczema. The findings suggest a possible rôle of the regional lymph nodes in the production of antibody-like factors. This problem, however, together with the mechanism of the allergic response of the skin (test) requires further study.

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For further bibliography see *Frey* und *Wenk* 1956

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The Action of Bacterial Polysaccharides on Allergic Phenomena

By R. MEIER, H J BEIN and R JAQUES

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Basle

Extensive work on the biological effects of bacterial and other polysaccharides conducted in our laboratories has shown that these substances are highly active in various respects. They exert a pronounced stimulant effect on leucocyte emigration (26, 31 a, b). Depending on the route of administration and on the type of bacteria, they inhibit or enhance the inflammatory response of the mesenchymal tissue (granuloma) following introduction of a foreign body (28). The polysaccharides may also favourably influence experimental bacterial or viral infections (22, 29, 30, 37, 38).

In a preliminary [illegible]

[illegible] tions were obtained from gram-negative bacteria and especially by fractionation of various *Proteus* species and culture filtrates of these organisms*. In the meantime, we have tested a large number of new polysaccharide preparations derived from *Proteus* and have also extended our investigations on their mechanism of action.

Anti-Allergic Properties

The ps were studied as to their effect on systemic as well as local anaphylaxis, using the following tests:

1. Protection against death from anaphylactic shock in guinea-pigs.

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* The ps were prepared by Dr. *F W Kahnt* in our Chemical Department.

themselves produce such factors. The latter postulation is partially confirmed by *Skog* who obtained positive transfer experiments using among other agents lymph fluid from the thoracic duct.

d) We do not know whether beside the regional lymph nodes other organs or systems also participate in the production of antibody-like factors. These processes may be looked for especially during the incubation period, *i.e.* during the first 5 days after the primary contact.

e) We consider that the factors responsible for the sensitization of the entire skin are spread by the blood path. It seems less probable that transmission occurs via the nervous system.

We believe that this study contributed certain links clarifying the pathogenesis of contact eczema. The findings suggest a possible rôle of the regional lymph nodes in the production of antibody-like factors. This problem, however, together with the mechanism of the allergic response of the skin (test) requires further study.

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For further bibliography see *Frey* und *Wenk* 1956.

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an intravenous injection of pyrogen-free saline. Identical lengths of the distal portion of the ileum were suspended in a 20 ml organ bath as described above. Exactly 30 minutes after inserting the intestine in the bath, threshold doses of diluted egg-white were added to the organ bath. The height of contraction of the control intestines was compared with that of animals which had received a ps injection prior to being killed. Separate groups of sensitised guinea-pigs were submitted to the same procedure, except that in this case the dose of the antigen was 50–100 times higher than the minimal effective dose.

Results

Certain preparations derived from *Proteus vulgaris* proved distinctly protective against death from anaphylactic shock. The minimum effective dose, i. e. the dose which when given 30–60 min. before induction of shock would reduce mortality from 100% to at least 50%, was found to range from 0.1–3 μg/kg in the case of the best preparations. The anaphylactic symptoms in the surviving guinea-pigs were reduced in intensity and duration according to the dose and depending on the preparation employed. With some preparations practically no symptoms at all were observed or, if so, the symptoms were usually only short-lived. Usually good ps reached their optimal anti-anaphylactic activity only 30–60 min. after the injection.

In those instances where the duration of effect of the ps was estimated, the protective action lasted about 4 hours with a dose of 3 μg/kg, whereas 10 times this dose afforded protection for at least 24 hours. Thus, the anti-anaphylactic activity proved distinctly superior to that of known antihistamines and was also of appreciably longer duration.

In contrast to synthetic antihistamines it was occasionally observed that ps preparations which had proved fully protective in previous tests failed to do so when re-tested under identical conditions but with different batches of sensitised guinea-pigs. The reason for this is as yet unknown.

It is noteworthy in this connection that, among the different ps preparations tested by us, the majority proved inactive in dosages of 0.1–3 μg/kg as far as their anti-anaphylactic effects were concerned. Increasing the dosage of an anti-anaphylactically ineffective ps 10–20 fold did not result in any measurable improvement in its anti-anaphylactic activity.

Anti-anaphylactically active preparations inhibited or suppressed the active Arthus reaction in rabbits or guinea-pigs. Doses of 10–50 μg/kg were necessary in the rabbit, whereas in guinea-pigs

2. Effect on the active Arthus reaction in
   a) the rabbit and
   b) the guinea-pig.
3. Effect on the anaphylactic reaction *in vitro*.

The methods employed were as follows:

1. Anaphylactic shock:

Male guinea-pigs with a body weight of 200–300 g were sensitised by a single intraperitoneal injection of 0.5 ml of fresh egg-white diluted 1 : 5 with physiological *saline or alternatively 0.5 ml of a 1% solution of ovalbumin. Three weeks later the* guinea-pigs were challenged with an intravenous dose of 0.2 ml of fresh egg-white diluted 1 : 100 or 1 : 50 with physiological saline or alternatively 0.2 ml of a 0.1% ovalbumin solution. This challenging dose regularly caused death in at least 85–90% of all our untreated controls. The ps preparations considered in this publication were, however, only tested on series of guinea-pigs in which 100% of the controls died. The ps preparations were dissolved in pyrogen-free distilled water and injected by the venous route. The usual interval between injection of the ps and subsequent challenging varied from 10 min. to 2 hours. Most of the tests were performed after an interval of 60 minutes. In a few instances, this interval was extended up to 72 hours in order to evaluate the duration of action of a given preparation.

2. Arthus reactions:

Rabbits of either sex weighing from 1.5–2 kg were sensitised by a course of three injections of 2 ml each of phenol-free horse serum. The first and third injections were given intraperitoneally and spaced two weeks apart. The second was administered subcutaneously on the day following the first intraperitoneal injection. A week after the final intraperitoneal injection, the Arthus reaction was elicited by intracutaneous injection of 0.5 ml undiluted horse serum into the clipped skin of the belly. The development of the reaction was recorded during 48 hours. In most cases, the ps were injected intravenously in one single dose one hour before the antigen.

b) Male guinea-pigs were sensitised by 3 intraperitoneal injections given on [illegible] serum diluted 1 : 1 with physiological [illegible] *(Armour)* in saline. In some experiments [illegible] Two weeks after the final intraperitoneal injection, the Arthus reaction was elicited by intracutaneous injection of 0.5 ml of the corresponding antigen (undiluted horse serum, 20% crystalline ovalbumin) into the clipped belly skin. The local changes were recorded during a period of 24–48 hours, after which the animals were sacrificed. Flaps of the belly skin were prepared and the severity of the reactions also estimated from the inner surface of the reflected skin.

3. Effect on anaphylaxis in vitro:

[illegible] with egg [illegible] Tyrode at [illegible] ited fresh egg-white, usually 0.2 ml of a 1 : 500 (v/v) dilution. The ps were added to the organ bath in final concentrations of up to 0.1 mg/ml 2–5 minutes prior to adding the antigen.

b) Groups of 10–16 male guinea-pigs sensitised as described under 1 were divided at random into 2 equal lots. One half received an intravenous injection of the ps 30–60 min. before being sacrificed, while the other half served as controls and received

Table I

In vitro anaphylaxis (Schultz-Dale reaction) of guinea-pigs treated *in vivo* with polysaccharide preparations*

Guinea-pig No	Treatment	Height of contraction mm	
930	Controls	44	
900	2cc saline/	46	
910	kg i. v.	54	mean: 51
914		74	
912		38	
922	Preparation	7	
951	No. 16617	2	
901		15**	mean: 12
911	3 µg/kg i. v. 30 min. be-	18**	
932	fore being sacrificed	18**	
602	Controls	52	
612	2cc saline/	39	
628	kg i. v.	70	mean: 50
631		48	
608		42	
607	Preparation	12**	
614	No. 17381/2	0	mean: 10
615		5	
610	3 µg/kg i. v. 60 min. be-	8 **	
623	fore being sacrificed	24**	

* Distal ileum suspended in Mg-free Tyrode; test conducted exactly 30 min. after insertion in organ-bath on pieces of identical length with treshold concentrations of fresh egg-white diluted with saline. Final dilution of egg-white 1 : 10 000

** Onset of *contraction* appreciably delayed as compared with controls

These experiments clearly show that under certain conditions the ps produce an anti-anaphylactic effect by altering the reactivity of the organ towards the action of the antigen. This action is present only where the amount of antigen falls within a defined range. The reaction therefore appears to be in some way a competitive one. As it does not seem to depend quantitatively on the amount of ps, a preliminary assumption might be made to the effect that the ps acts on a body constituent responsible for anti-anaphylactic protection, the amount of which determines the quantitative competition to the antigen/substrate reaction; this question will have to be investigated in detail, since it may well lead to a closer understanding of the anaphylactic mechanism.

In order to define this mechanism of action in other directions it seemed important, firstly to determine the relation of the ps effect to other tissue components which are important in anaphylaxis such as histamine, serotonin, etc. and secondly to relate the

as little as 1–3 μg/kg was sufficient to suppress the reaction completely. This inhibitory effect was likewise observed in guinea-pigs which had been sensitised with *Freund's* adjuvant mixture (antigen + killed BCG + mineral oil), where the usual reaction is characterised by intensive haemorrhage followed by necrosis, the oedema being considerably less marked than in guinea-pigs sensitised without adjuvant.

A dissociation between the anti-anaphylactic and anti-Arthus properties of ps preparations was observed inasmuch as some of the ps which were inactive as regards their anti-anaphylactic effects in the guinea-pig in a dose of 3 μg/kg nevertheless inhibited the Arthus reaction in either guinea-pig or rabbit in doses of 3 and 10–50 μg/kg respectively.

When a ps which injected 1–3 hours before the antigen had proved fully effective in rabbits, was injected 24 hours before the antigen, either no inhibitory effect was observed at all or, following initial inhibition there was sometimes even an aggravation of the haemorrhagic and necrotic reaction.

For further characterisation of the action and its mechanism it seemed necessary to determine whether ps active *in vivo* possess an effect on anaphylactic phaenomena similar to that of antihistamines.

No effect was noted on the Schultz-Dale reaction of the guinea-pig's ileum when the ps was added *in vitro* to the sensitised intestine. The interval between addition of the ps and the antigen as well as the dosage of the ps varied over a considerable range, but in no case was this found to have any appreciable effect on the anaphylactic contraction. It seemed for various reasons possible that the ps given *in vivo* might alter the anaphylactic reactivity of the organs of sensitised animals in such a way that the *in vitro* reaction would be appreciably diminished. The isolated intestine of sensitised animals which had received an intravenous injection of a ps 30–60 min. prior to being sacrificed was distinctly less sensitive to threshold concentrations of the antigen. When the concentration of the antigen was increased 50–100 times above the minimum effective dose, the difference observed in this *in vivo / in vitro* test as between controls and polysaccharide-treated animals disappeared. Table I gives two examples of combined *in vivo / in vitro* experiments performed with different ps preparations and using threshold antigen concentrations.

Binding of Histamine in vitro

a) The ps and histamine dihydrochloride were dissolved separately in distilled water or saline in concentrations of 1–10 μg/ml or 10–100 μg/ml respectively. Equal volumes of these solutions were thoroughly mixed in a test tube and compared with histamine solutions using the isolated guinea-pig ileum or the blood pressure of the atropinised cat as test objects.

b) *Werle's technique (47)* The ps were dissolved in 1% acetic acid containing 2 mg histamine/6 ml in concentrations varying from 33 μg – 330 μg/ml

6 ml aliquots of these solutions were dialysed at room temperature for 2 hours against a known volume (194 ml) of 1% acetic acid. The histamine content of these dialysates was compared with that of controls in which no ps was present in the cellophane bag Control experiments were also conducted using heparin in concentrations of 0 33 to 1.33 mg/ml

Results

Some of the ps, when mixed *in vitro* with histamine in a ratio of 1 : 100 and using final concentrations of the ps of up to 1 μg/ml, were found to bind part of the histamine, as evidenced by tests on the isolated guinea-pig ileum or on the blood pressure of the atropinised cat. This effect was discernible almost immediately after mixing, but did not increase – even in response to prolonged incubation at room temperature.

When the concentration of ps was stepped up, there was no appreciable increase in the binding of histamine; on the contrary the binding effect sometimes even disappeared completely. No binding of histamine was observed using Werle's dialysing technique (47) with rather high concentrations of ps and histamine. It should be noted, however, in this connection that in most experiments the ps were employed in concentrations 10 times inferior to effective concentrations of heparin.

Determination of *Shwartzman* reactivity seemed to offer an interesting possibility of demonstrating a connection with other types of immunological response, although it may not help greatly to explain the anti-anaphylactic action of ps until more is known about the extent to which this *Shwartzman* reactivity is a reliable index for such types of effect.

Shwartzman Phenomenon

Rabbits were injected intracutaneously with [illegible]
50 [illegible]
C [illegible]
d [illegible]
[illegible] second i v. dose of 10–30 μg/kg

anti-anaphylactic effect to other types of action displayed by ps. The following selected tests have been used for this purpose.

Antihistamine and Antiserotonin Activity in vitro and in vivo

a) In vitro

Pieces of the distal ileum of guinea-pigs were suspended in the organ-bath in the usual manner and perfused with oxygenated Tyrode. The ps preparations were added to the organ-bath in final concentrations of up to 0.1 mg/ml and left in contact with the organ for 2–5 min prior to the addition of histamine. In a manner identical with that used for the mixed *in vivo / in vitro* anaphylaxis test, guinea-pigs were injected i. v. with up to 100 times the anti-anaphylactic dose and their gut subjected to histamine contractions The ps under test were also added *in vitro* to the isolated rat colon suspended in Ca–low perfusing fluid (19) and tested for possible direct antiserotonin effects.

b) In vivo

Guinea-pigs were injected i. v. with up to 10 times the anti-anaphylactic dose of a given ps Starting ½ hour after the administration of the ps and continuing for 24 hours, the response to the action of a 0 5% histamine aerosol was tested Using *Herxheimer*'s technique (14, 15), serotonin (5-HT) was administered as a 2% aerosol; since guinea-pigs show pronounced hyporeactivity to serotonin upon repeated exposure, separate lots of guinea-pigs were used in order to evaluate the effect at various intervals after the i. v. administration of the ps

Results

No direct antihistamine effect was found in the isolated guinea-pig gut, nor was the histamine sensitivity of the gut of guinea-pigs treated with ps *in vivo* altered to any appreciable degree. Similarly, treatment with ps afforded guinea-pigs no protection against histamine aerosol. On the other hand, some slight protection against broncho-constriction and coma resulting from exposure to serotonin aerosol was observed with doses which protected against anaphylactic shock. No antiserotonin effect was noted, however, when the ps were added *in vitro* to the isolated rat colon.

Therefore, the antagonistic effects on these stimulants are either absent altogether or, if present, only negligible; they probably play no major role in the anti-anaphylactic effect of ps.

In spite of the negative results obtained with ps against histamine *in vitro* or *in vivo*, it seemed possible that some effects of ps might be traced using other methods. Among these effects, the binding of histamine – which takes place with certain ps such as heparin (47) – seemed of particular interest and was therefore investigated using two different methods.

Binding of Histamine in vitro

a) The ps and histamine dihydrochloride were dissolved separately in distilled water or saline in concentrations of 1–10 μg/ml or 10–100 μg/ml respectively. Equal volumes of these solutions were thoroughly mixed in a test tube and compared with histamine solutions using the isolated guinea-pig ileum or the blood pressure of the atropinised cat as test objects.

b) *Werle's technique (47).* The ps were dissolved in 1% acetic acid containing 2 mg histamine/6 ml in concentrations varying from 33 μg – 330 μg/ml.

6 ml aliquots of these solutions were dialysed at room temperature for 2 hours against a known volume (194 ml) of 1% acetic acid. The histamine content of these dialysates was compared with that of controls in which no ps was present in the cellophane bag. Control experiments were also conducted using heparin in concentrations of 0.33 to 1.33 mg/ml.

Results

Some of the ps, when mixed *in vitro* with histamine in a ratio of 1 : 100 and using final concentrations of the ps of up to 1 μg/ml, were found to bind part of the histamine, as evidenced by tests on the isolated guinea-pig ileum or on the blood pressure of the atropinised cat. This effect was discernible almost immediately after mixing, but did not increase – even in response to prolonged incubation at room temperature.

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Determination of *Shwartzman* reactivity seemed to offer an interesting possibility of demonstrating a connection with other types of immunological response, although it may not help greatly to explain the anti-anaphylactic action of ps until more is known about the extent to which this *Shwartzman* reactivity is a reliable index for such types of effect.

Shwartzman Phenomenon

Rabbits were injected intracutaneously with doses of [illegible] 50 μg, 24 hours la[illegible] Other groups of [illegible] the preparatory [illegible] i.v. dose of 10–30 μg/kg

Results

When tested on rabbits using *Shwartzman*'s technique (40, 41), all the ps so far examined showed preparatory as well as provocative properties. A simultaneous i. v. injection of one and the same ps along with the preparatory injection was capable of greatly reducing the intensity of the local reaction. Furthermore there were fewer macroscopically detectable non-dermal lesions of the Sanarelli type in such animals.

In elucidating the anti-anaphylactic action of ps it might be important to determine the connection with the other effects which they produce – such as the pyrogenic effect and the action on leucocytes. These latter seem to be of same significance in this connection and must be taken into consideration in addition to the effects already mentioned.

Pyrogenicity, Leucocyte Response

Most experiments on pyrogenicity were performed on guinea-pigs or rabbits A few were carried out on cats anaesthetised with dial-urethane or chloralose The ps was injected i. v in doses ranging from 0 001 to 100 μg/kg and the rectal temperature recorded for periods of up to 24 hours Similarly, at various intervals leucocyte counts were made of blood taken by heart puncture in guinea-pigs, from the marginal ear vein in rabbits, or from the exposed femoral artery in cats Differential white cell counts, including thrombocyte counts, were made from blood smears

Results

The increase in body temperature which follows injection of bacterial ps, especially ps derived from gram-negative bacteria, is the most widely known effect of these substances (4, 5, 43, 48).

It is generally believed that the pyrogenic reaction is not a primary effect and, furthermore, that the hyperthermia is in some way preceded by and dependent upon the leucopenia which follows injection of bacterial ps Non-anaesthetised rabbits and cats anaesthetised with dial-urethane, exhibited a pronounced rise in body temperature which usually set in after a typical latency of between 20–40 min. following the injection and which returned to normal within 3–4 hours when threshold pyrogenic doses or doses of up to about 30 times the threshold dose were employed. With higher doses the hyperthermia lasted longer and, in certain instances where it was followed up, was still detectable after as much as 10–12 hours. Still higher doses sometimes caused a fall in body temperature, probably as a result of toxic or circulatory effects.

The majority of the ps studied proved highly pyrogenic; in fact, when injected intravenously into guinea-pigs, some of them still produced a significant rise in rectal temperature in a dose as low as 0 001 μg/kg. On the other hand, taking 3 μg/kg as the critical dose, some ps were found to be inactive in so far as they produced no pyrogenic reaction in the guinea-pig; some of these preparations nevertheless proved effective against anaphylactic shock. Increasing the dose of such ps 10 fold or more usually resulted in the appearance of mild to moderate hyperthermia, but with no appreciable alteration in the anti-anaphylactic effect.

Following injection of a ps in a definitely pyrogenic dose, a pronounced fall in total circulating leucocytes occurred within 5–10 min, i. e. earlier than the rise in rectal temperature. The leucopenic effect was often spectacular in that as much as up to 85% of the leucocytes disappeared from the peripheral circulation. It was also noted that during the leucopenic phase the buffy coat taken from heparinised blood tended to be more sticky than at other times. As a rule, the leucopenic phase was followed by leucocytosis, which began within 3–4 hours after the intravenous injection of the ps and attained a maximum exceeding the preexisting normal level by as much as 70–80%.

The leucocytosis was due to polymorphonuclear cells and usually lasted several hours before returning to normal levels; it was accompanied by more or less pronounced lymphocytopenia. The platelets fell within about 15 min following injection of the ps and remained at subnormal levels for several hours These haematological effects and the hyperthermia were observed with some variation in degree and quality in all the species examined. The non-anaesthetised rabbit with its normal lymphocytosis showed the most marked polymorphonuclear leucocytosis and lymphopenia, whereas in the anaesthetised cat these effects were appreciably less pronounced. Typical examples of these responses are shown in figures 1 and 2

Influence of Anaesthesia on Pyrexia and Leucocyte Response

As an interesting chance-observation, we found that in cats ps produced a pronounced rise in body temperature, together with the leucocyte fluctuations described, when dial-urethane was

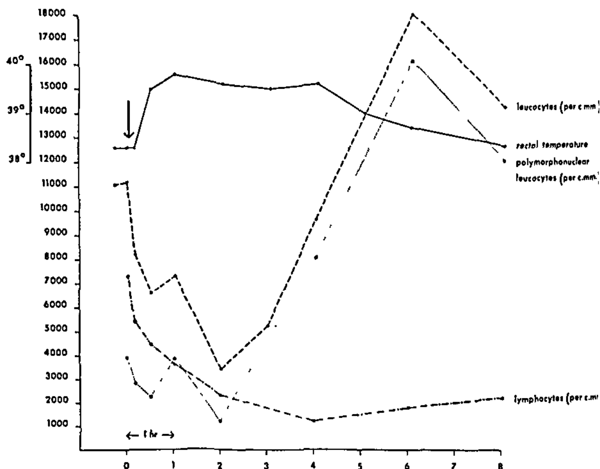


Fig. 1. Rabbit ♂ 2 0 kg. At arrow intravenous injection of the ps preparation No 16617 3 μg/kg

Table II

Influence of anaesthetic upon pyrogenic and leucocyte reactions in the cat

Anaesthetic	Polysaccharide preparation No	Dosage μg/kg	Rise in rectal temperature	Primary leucopenia secondary leucocytosis
Dial-urethane	16617	3	+++	+++
chloralose	«	3	+	+++
Dial-urethane	19338/8	0,03	+++	+++
chloralose	«	0,03	(+)	+++
Dial-urethane	19515/3	0,03	+++	+++
chloralose	«	0,03	(+)	++

employed as the anaesthetic, whereas when chloralose was used the same doses did not markedly affect body temperature although they still caused similar, if somewhat less pronounced leucocyte reactions (Table II).

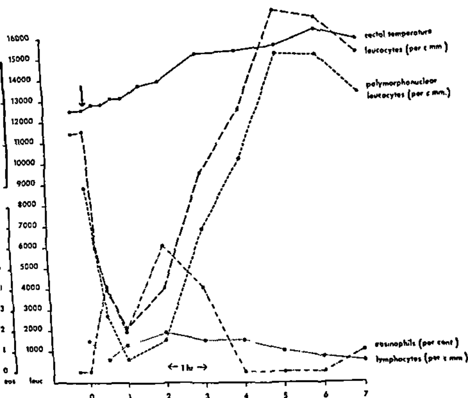


Fig 2 Cat ♂ 2 4 kg Dial urethane anaesthesia. At arrow intravenous injection of the ps preparation No 16617 100 μg/kg

Relationship Between Anti-Allergic and Pyrogenic Properties

Most of the ps had a pyrogenic effect on guinea-pigs when given in a dose which afforded protection against anaphylactic shock. But, as already mentioned, we have found a certain number of ps derived from *Proteus* which were non-pyrogenic when administered in a dose which protected guinea-pigs against death from anaphylactic shock. On the other hand, the pyrogenic preparations were often ineffective against anaphylactic shock, although they sometimes displayed an extremely potent pyrogenic activity in the guinea-pig.

It was also found that, when ps which were highly pyrogenic but inactive against anaphylaxis were given in a dosage so low as to have little pyrogenic effect, this did not result in the appearance of anti-anaphylactic properties in a preparation which had

failed to display them in a highly pyrogenic dose. Generally the doses necessary to produce sustained inhibition of the active Arthus reaction in the rabbit were some 10–20 times higher than those active against anaphylactic shock in the guinea-pig, and they usually produced a rise in body temperature lasting several hours. In guinea-pigs sensitised with either horse serum or ovalbumin, the doses which effected a clear-cut reduction in the intensity of the Arthus reaction were of the same order as those active against anaphylactic shock; those preparations which were active against anaphylactic shock in non-pyrogenic doses also inhibited the Arthus reaction in the same dose. On the other hand, a number of pyrogenic preparations which were inactive in the anaphylactic shock test showed good inhibitory activity against the active Arthus reaction in either rabbits or guinea-pigs. The complex pattern of these various activities is best illustrated in table III.

Table III

Pyrogenicity and anti-allergic effects of different ps

Polysaccharide preparation No.	Pyrogenicity Guinea-pig	Pyrogenicity Rabbit	Anti-anaphylactic activity *in vivo**	Schultz-Dale reaction "*in vivo/in vitro**"	Schultz-Dale reaction *in vitro*	Inhibition of Arthus reaction Guinea-pig*	Inhibition of Arthus reaction Rabbit**
16617	+	+	+	+	O	+	+
16619	+	+	+	+	O	+	+
18879/3	O		+	+	O	+	
19338/8	+++	++	+			+	+
19342/5	O		O			(+)	
19515/3	++	++	O		O	+	+

* Standard dose 3 μg/kg ** Standard dose 50 μg/kg

O – +++: Arbitrary activity units (applicable only to pyrogenicity)

All these observations show, that bacterial ps may produce different effects depending on the ps under study. Some derivatives may produce the whole gamut of effects, while others may exhibit only one of the possible properties. This indicates that a certain degree of specificity may perhaps be dependent on the particular chemical configuration of a given ps.

Discussion

It is generally accepted that histamine plays an important rôle in the processes of anaphylactic phenomena; similarly, serotonin – at least under certain conditions – is released during the antigen-antibody reaction (17, 18).

Since the ps active against anaphylactic shock or local anaphylaxis do not possess any detectable antihistamine activity *in vitro* or in the intact animal and since their antiserotonin activity *in vivo* is only feeble, it follows that such effects cannot contribute in any appreciable degree to the anti-anaphylactic activity of *Proteus* polysaccharides; their anti-allergic properties must necessarily reside in some other mechanism of action.

A direct mechanism, such as an inhibitory effect on vascular permeability due to «proofing» of permeable pores, can be ruled out *a priori* for purely quantitative reasons. As evidenced by our experiments, the binding of histamine *in vitro*, i. e. neutralisation of histamine in some way or other occurs only under certain particular conditions but not under conditions in which, for example, heparin is highly active. Furthermore, so far as we have been able to detect, ps injected intravenously have no significant influence on plasma histamine in anaesthetised cats. The antiserotonin activity of *Proteus* ps must be considered as an indirect effect, since it is not detectable when the ps are added *in vitro* to the isolated rat colon.

It is therefore most likely that the mechanism by which *Proteus* ps exert their anti-allergic effects differs from that of known anti-allergic drugs; furthermore, these effects do not appear immediately, since even when the ps are injected into the blood stream, some time elapses before any anti-allergic activity is detectable. Hence, it seems necessary, to elucidate the mechanism of action of *Proteus* ps in a study of the other reactions, including secondary ones, which may possibly follow injection of ps.

In 1955, *Humphrey* (16), using a pyrogenic ps fraction from *Chromobacterium prodigiosum*, found that it completely inhibited the Arthus reaction in rabbits; but he discarded this effect as due to non-specific interactions such as lowering of the blood pressure etc. Our experiments with ps obtained by fractionation of *Proteus vulgaris* barteria or culture filtrates provide evidence that ps in µg amounts are able to inhibit certain effects of the antigen-antibody reaction occurring in systemic anaphylaxis in the guinea-pig as well as local anaphylactic reactions of the Arthus type. Under certain conditions they also inhibit the *Shwartzman* phenomenon, for which they are used both as preparatory as well as provocative agents.

It has been assumed that an anti-anaphylactic action might be produced by elevation of the body temperature, by leucopenic reaction, and perhaps by other general non-specific reactions (16, 32). Ps invariably exert an effect on circulating leucocytes, but they need not necessarily be pyrogenic when given in a dose which affords protection against anaphylactic shock in the guinea-pig. It is thought that their effect on circulating leucocytes merely reflects one of the detectable reactions of the organism to unknown alterations in intrinsic reactivity rather than the primary condition for their anti-allergic activity. This interpretation would incidentally seem justified in view of the fact that a single dose producing transient granulocytopenia followed by polymorphonuclear leucocytosis with lymphocytopenia is able to suppress the Arthus reaction in guinea-pigs and rabbits for as long as 48 hours, whereas the leucocyte fluctuations take place within the first hours after injection of the ps.

A decrease in blood pressure and changes in the systemic haemodynamic condition of the anaesthetised animal occur irregularly and only in response to doses far in excess of those active against anaphylactic shock. Moreover, lowering the blood pressure with hydrazinopthalazines or ganglion blocking agents, for example, does not inhibit to any measurable degree either the anaphylactic shock in guinea-pigs or the Arthus reaction in rabbits and guinea-pigs. Thus, a reduction in blood pressure can be ruled out as a significant component of the anti-allergic properties of *Proteus* ps. It has repeatedly been reported that certain pyrogenic ps act by stimulating the pituitary-adrenal system (3, 5, 6, 7, 8, 25, 36, 39, 43); it is therefore conceivable that the bacterial ps which we have studied might act *via* such a mechanism, e. g. through the liberation of endogenous adrenomedullary and adrenocortical factors. Such a possibility might be worth considering in view of our findings that *isopropyl*-noradrenaline, which is a normal constituent of the adrenals and of bronchial sympathin (23, 24), exhibits marked antiserotonin and anti-anaphylactic activities in the guinea-pig (20) and that certain cortical steroids protect guinea-pigs against anaphylactic shock. It seems unlikely that ps derived from *Proteus* are able to provoke the release of such amounts of endogenous steroids; however, on the basis of the present experimental data, the possibility that steroids and/or substan-

ces like *isopropyl*-noradrenaline contribute to the antiallergic effects of *Proteus* ps is one that cannot be ignored.

Since there appears to be a parallel with other findings relating to the effect of ps on the immune reaction, their influence on the *Shwartzman* phenomenon seems interesting. Our findings that *Proteus* ps possess preparatory as well as provocative properties when tested according to *Shwartzman's* technique (40, 41) are in line with previous reports on such properties of bacterial and other ps preparations or materials containing ps (2, 5, 13, 42, 43, 45). It is noteworthy that the minimum doses necessary for preparing and provoking the *Shwartzman* phenomenon are of the same order of magnitude as those of a ps obtained by *Shear* from *Serratia marcescens* (42). In 1952, *Alechinsky* reported that culture filtrates of *Coli*, when injected intravenously at the same time as the preparatory intracutaneous dose, largely inhibited the severity of the local *Shwartzman* phenomenon following provocative intravenous injection of one and the same filtrate (1). We have now been able to confirm these results using small doses of our purified ps preparations.

There is also a possibility that the anti-anaphylactic effect occurs in connection with the phenomenon of «promunity». In 1936, *Ørskov* and *Kauffmann* (33) found that mice which had received dead typhoid bacilli i. v. or i. p. developed a resistance to lethal doses of this organism 1–3 days after administration. Similar findings have recently been published by *Rowley* (37, 38) and others (22). This initial resistance disappeared rapidly and was not due to classical specific immunity, since following the injection of one species of dead Gram-negative bacillus transient protection against many other species was obtained. This phenomenon can also be provoked by highly purified ps of Gram-negative bacteria, gonadotrophic hormone fractions, etc. as described by *Meier* et al. (30). It may be that this phenomenon of "promunity" is connected with *Pillemer's* properdin system (34, 35, 46). But on the basis of the present evidence, it cannot be assumed that such phenomena as «promunity» and the properdin system are the main or only factors responsible for this type of reaction In any case, they may have a common denominator with the anti-allergic effects observed by us using ps from *Proteus*.

Thomas and coll. (44) have shown that, when high doses of a ps derived from *Serratia marcescens* are injected into rabbits, they

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They have no direct antihistamine or antiserotonin effect *in vitro*, nor do they counteract the effects of histamine aerosol in the intact animal Their antiserotonin activity in the intact guinea-pig exposed to serotonin aerosol is feeble. Under certain conditions they bind histamine *in vitro*, but do not cause any appreciable change in plasma histamine in anaesthetised cats. Most of them are pyrogenic in the doses in which they afford protection against anaphylactic shock. However, some of them are non-pyrogenic in anti-anaphylactic doses; thus pyrogenicity is not a prerequisite of their anti-anaphylactic property, nor does their anti-allergic activity seem to be directly correlated with their effects on leucocytes The only effect directly connected with the anti-anaphylactic action which we could find in our experiments was that the reactivity of the isolated gut taken from an animal treated with anti-anaphylactic doses of ps, is greatly reduced when threshold concentrations of the antigen are used although it reacts normally towards histamine. The mechanism of this effect is unknown, but would seem to represent a new type of anti-anaphylactic action.

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cause the appearance in the plasma of a protein which can be precipitated at a low temperature in the presence of heparin. The ps studied by us produced no such effect in rabbits even when injected in doses higher than those causing full inhibition of the Arthus reaction.

When ps derived from *Proteus* are added *in vitro* to the intestine of sensitised guinea-pigs, they do not affect its reaction upon addition of the antigen. However, when the ps are injected *in vivo* into sensitised guinea-pigs, the intestine taken from such animals is distinctly less sensitive to threshold concentrations of the antigen, although it still reacts to histamine in a manner identical to that of the intestine obtained from non-treated sensitised animals. This clearly shows that these ps derived from *Proteus* can profoundly change the anaphylactic reactivity of a smooth-muscular organ without altering its sensitivity to histamine, and that they do so under conditions in which no humoral antianaphylactic agent or any effects involving the leucocytes (16), temperature (32), blood-pressure (16), etc. can be operating directly. It is therefore possible that such ps exert their anti-allergic effects in the intact animal either by directly or indirectly changing some cellular constituent(s) involved in the antigen-antibody reaction or by causing the release of some factors which become fixed to the cell, thus rendering it less sensitive to the effects of the anaphylactic reaction.

It is not known whether any of the factors mentioned change the reactivity of the anaphylactic organ after the animal has been treated. This question is certainly of paramount importance, as it may have a bearing on other problems of immunisation and sensitisation.

Our experimental evidence is so far obviously insufficient, and cannot completely explain the more intimate processes underlying the antiallergic effect of our bacterial ps. These ps most certainly do not exert their effect directly in the manner of classical anti-allergic drugs and further work will have to be done in order to elucidate their mechanism of action.

Summary

Ps derived from cells of *Proteus vulgaris* or culture filtrates of this organism possess anti-allergic properties in doses which are by far inferior to those of other known anti-allergic drugs.

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On Anaphylatoxin

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In 1910, in the same year as *Dale* and *Laidlaw* described the similarity between histamine intoxication and anaphylactic shock, *Friedberger* prepared anaphylatoxin. At first this attracted more attention from anaphylaxis research workers. Perhaps it was advantageous that the anaphylatoxin theory tried to explain the connection between the antigen-antibody reaction and the toxin formation experimentally. As is well known, *Friedberger* produced the toxin by contact between guinea-pig serum and immune precipitates. But the interest in anaphylatoxin still remained after both *Bordet* and *Nathan* had obtained anaphylatoxin with polysaccharides instead of immune precipitates, and after *Novy* and *De Kruif* had obtained anaphylatoxin by simply diluting the serum with distilled water. On the other hand, interest suddenly vanished when *Dale* and *Kellaway* in 1922 demonstrated that the properties of an anaphylactic toxin were only incompletely represented in anaphylatoxin. On the isolated uterus of a guinea-pig, they obtained only weak and irregular contractions, whereas under the stimulation of an antigen-antibody reaction, as also under the influence of histamine, this organ showed strong contractions. It therefore became doubtful whether the lethal effect of anaphylatoxin in the intact animal is connected with a direct effect on the smooth musculature, or whether it actually leads to a fatal bronchospasm. When 10 years later *Feldberg* led the histamine theory to triumph, he no longer had to fight against the resistance of the anaphylatoxin theory. During the last few years, however, interest in anaphylatoxin has been aroused once more, since it was found that it possesses the properties of a histamine liberating substance. As we showed

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together with *Oberdorf* in 1951, antihistamines prevent fatal anaphylatoxin shock. A rise in histamine takes place in the plasma *(Hahn)*. This increase is parallel to the severity of the shock. The isolated guinea-pig lung also liberates considerable quantities of histamine when flooded with anaphylatoxin. This was demonstrated by *Rocha e Silva* in pursuance of our examinations.

There can therefore be no further doubt that death in anaphylatoxin shock is to a great extent due to the bronchospasm caused by histamine. The effect of anaphylatoxin on isolated smooth muscle organs was improved by *Rocha e Silva* when he used rat serum anaphylatoxin, which is 4 times stronger than the anaphylatoxin obtained from guinea-pig serum.

Apart from this, it can be deduced from the experiments of *Dale* and *Kellaway* that even anaphylatoxins from guinea-pig serum do not completely lack the contracting effect on the isolated uterus, particularly if such toxins are obtained with agar instead of starch. Also in the lung of intact animals, such anaphylatoxins produce syndromes which are more typical of anaphylaxis.

More frequently, rat serum anaphylatoxin produces a syndrome resembling that of pure pulmonary emphysema without oedema and haemorrhages. Thus, the more effective the anaphylatoxin, the clearer and more undiluted are the symptoms caused by the histamine action. Here also, anaphylaxis and anaphylatoxin shock may perhaps resemble each other, since a subacute anaphylactic shock is accompanied to a great extent by haemorrhages and oedema than by bronchospastic effects.

Dale and Kellaway were able to overcome the bronchiolar resistance in intact animals by artificial respiration more easily in anaphylatoxin shock than in anaphylactic shock. If one attempts to explain this observation by the low efficacy of the guinea-pig serum anaphylatoxins used, then one encounters the paradox that *Rocha e Silva* obtained, with the same type of anaphylatoxin, a much larger histamine yield from the isolated lung than through an antigen-antibody reaction, according to the experiments of *Feldberg* and *Schild*. Recently, *Dale* particularly emphasised this point and sees in it a further proof for a more qualitative than quantitative distinction. Under the effect of anaphylatoxin, the liberation of histamine is supposed to be located more at the endothelia, whereas in anaphylactic shock it takes place more at the smooth muscles. It should not be overlooked that here the hista-

mine liberation in the isolated lung rinsed with Tyrode's solution is related to the pulmonary inflation of intact animals. In intact animals, the histamine liberation in anaphylactic shock would appear to be rather stronger than after the injection of guinea-pig serum anaphylatoxin, which would also correspond to the findings of *Dale* and *Kellaway*. This is seen if our blood findings in anaphylatoxin shock are compared to the results obtained by *Code* in anaphylaxis. Unfortunately, the blood values cannot be so well evaluated, since in shock, a decrease in leukocytes and blood platelets takes place. Through this phenomenon, it is even possible that a decrease in the blood histamine values can be observed despite an increase in the plasma. We are, at present, carrying out comparative experiments with plasma. It also seems necessary to re-evaluate the histamine liberation of isolated lungs during anaphylactic reaction and anaphylatoxin shock in a strong comparative manner. *Ungar* has reported that the isolated lung, when flooded with serum, gives off more histamine in anaphylactic shock than with serum-free lavage.

Rocha e Silva has repeatedly discussed the considerations which indicate the participation of a humoral factor in anaphylactic shock, and which would explain the recent interest in anaphylatoxin research. Further research is necessary in order to show whether the humoral factor is identical with anaphylatoxin.

Recently we have attempted to obtain an indication as to the part played by anaphylatoxin in anaphylactic shock, by examining whether any decrease in the capacity of the serum to form anaphylatoxin takes place in anaphylactic shock. So far these experiments have only been concluded with inverse shock. We tested this form of shock first, because here the participation of a humoral factor is particularly evident, and because this shock can be reproduced constantly, and as often as is required. In guinea-pigs, we injected a constant dose of a haemolytic sheep's blood amboceptor About one minute later, we took blood from the carotid Together with dextran, the serum obtained from this blood gave an anaphylatoxin which was at an average less than half as potent as that obtained from normal serum. Coagulation in the shock blood, especially retraction of the blood cake, was delayed We therefore devised various experiments in order to determine whether the formation of anaphylatoxin was influenced by this circumstance. Since in shock the blood platelets and leuko-

cytes are reduced, shock plasma (oxalate plasma) was coagulated together with normal blood sediment (through recalcification), whereby the formation of anaphylatoxin was not increased. Prevention of the coagulation of normal blood through heparin in small doses did not reduce the formation of anaphylatoxin. The protein content in the serum of inverse shock does not decrease, but rather increases. Thus, there is no diluting factor present. The findings permit the conclusion that in inverse shock a certain protein fraction which plays a part in the formation of anaphylatoxin, is consumed. Together with *Giertz* we demonstrated the release of histamine in inverse shock. But perhaps the oedema-producing component of anaphylatoxin also plays a part here.

In high doses, heparin inhibits the formation of anaphylatoxin. It then also inhibits inverse shock, but hardly anaphylatoxin shock. Whether there are any connections here is a matter on which we can only conjecture.

It is certain that we will be able to say more about the biological significance of anaphylatoxin, when we have more accurate data on its chemical nature and formation. The purpose of our experiments is to obtain information with the method of *Cohn* on the serum factors involved in the anaphylatoxin effect and anaphylatoxin formation through the fractionation of rat serum. If rat serum with an anaphylatoxin content is fractionised, then anaphylatoxin is only found in the albumine-free fractions I–II–III and I–III, although the efficacy in these fractions is reduced by half. With further separation, the majority is found in III–0, while a residue remains in I–III–1, 2, 3. III–0 is obtained from I–III through extraction and re-precipitation. If extraction is carried out twice, then in III–0 not more anaphylatoxin is obtained than with a single extraction process, although the protein content is twice as high. The anaphylatoxin effect of III–0 is about $^1/_3$rd of that of the total serum, while the protein content is $^1/_{10}$ or $^1/_5$ of the total serum protein, according to the extraction. Thus, in relation to the protein content, in III–0 an appreciable accumulation of anaphylatoxin is to be observed.

In the III–0 fraction from incubated serum, the protein content is somewhat higher than that in the III–0 fraction from normal serum, while in I–III–1, 2, 3 it is reduced by about the same extent. The protein variation amounts to about 1.5% of the total serum protein. This protein which transfers into the

III–0 fraction may, together with a factor contained in III–0, form the anaphylatoxin.

If the fractions from normal serum are isolated and subsequently incubated with dextran, then an anaphylatoxin effect is obtained with I–III, but not with III–0 or I–III–1, 2, 3 alone, provided that both fractions are separated exactly through double extraction of I–III. If they are then mixed again, an anaphylatoxin is obtained which has an efficacy not much less than that of the incubated native serum. With incubation of one hour, the efficacy equals about $^2/_3$ of that of the initial serum, with a protein content of 36 % of the total serum protein.

We thus succeeded in demonstrating that two sharply separable factors play a part in the formation of anaphylatoxin. This rendered it possible for us to examine a few questions of reaction kinetics. I would like to summarise the most important results, which were obtained partly through using the isolated factors, and partly with native serum

1. The anaphylatoxin formation takes place with a measurable speed. This speed decreases by more than half on lowering the reaction temperature by 10° C.

2. Through diluting the serum with Tyrode's solution (1 : 4), the absolute anaphylatoxin formation is hardly influenced.

3 If the mixture ratio between III–0 and I–III–1, 2,3 is altered as compared to the normal ratio in such a manner that either III–0 or I–III–1, 2, 3 is reduced by a half or a quarter, then the anaphylatoxin formation is reduced (with constant incubation time). This reduction is in the average somewhat lower than that which would correspond to the degree of dilution.

4 If one factor (III–0 or I–III–1, 2, 3) is obtained from incubated serum and the other from normal serum, then no further formation of anaphylatoxin takes place (with constant incubation time). Thus, in the formation of anaphylatoxin, a consumption of both factors takes place.

5 The factor contained in III–0 is sensitive to a temperature of 56° C. This is not the case with the factor contained in I–III–1, 2, 3, nor with anaphylatoxin itself. Lower temperature degrees have not been examined.

6. The factor contained in III–0 is sensitive to zinc. This is not the case with the factor contained in I–III–1, 2, 3 or with anaphylatoxin itself Other metals have not yet been tried.

with the inhibition of an anaphylactic process in the skin. However, it does not appear that the process is associated with histamine-reactions. On the other hand, we found that a certain type of anaphylactic process leads to a decrease of the formation of anaphylatoxin in the serum.

The liberation of histamine by anaphylatoxin or by anaphylactic processes proceeds in a different manner than by low-molecular substances (for instance 48/80 or octylamin).

The inhibition of the anaphylatoxin formation by zinc-ions may be due to the affinity of the zinc to SH-groups. In this connection it is of interest to know that iodine acetate, another SH-group poison, may inhibit the liberation of histamine by conveyance of antigens to a sensitized organ *(Schild)*. Provided that the anaphylatoxin participates in the anaphylactic events, this finding may be explained by an inhibition of the anaphylatoxin formation as well as by inhibition of its effect. In fact, *Moussatché* observed that typical poisons of the SH-group are capable of inhibiting the effect of anaphylatoxin and of antigen on sensitized organs.

No unambiguous answer can be given regarding the mechanism of the histamine liberation by anaphylatoxin and the antigen-antibody reaction. The theory of ferment activation by *Rocha e Silva*, and especially by *Ungar*, is in contradistinction to some important arguments *(McIntire)*. The liberation of histamine by tryptic ferments is obviously only one of the known possibilities of this process *(Paton)*.

Now as before, the defenders of the anaphylatoxin theory of anaphylaxis face the difficulty regarding the possibility of an anaphylatoxin formation on isolated organs which can show an anaphylactic reaction. *Rocha e Silva* in this connection discusses the formation of anaphylatoxin in intercellular fluids during the anaphylactic process.

Perhaps further investigation will show that there is no anaphylatoxin-like humoral factor which participates in the anaphylactic shock of guinea-pigs. But the possibility remains that the process of anaphylatoxin formation in vitro is a model for what is going on in the tissues during anaphylactic shock, the tissue proteins perhaps playing the rôle of the serum proteins in vitro.

In any case, the fact that the body's own protein is able to transform into a histamine liberating substance by relatively trifling

measures is a biologically interesting phenomenon and deserves further attention.

Summary

Anaphylatoxin has the properties of a histamine liberating substance for the guinea-pig. The question of its participation in anaphylaxis still remains unanswered. Recently it has been demonstrated that in inverse anaphylactic shock there is a decrease in the capacity of the serum to form anaphylatoxin. The mechanism of anaphylatoxin formation *in vitro* has been further elucidated. The process depends on the presence of two serum factors which can be separated by *Cohn's* method of fractionation. The formation has a high temperature coefficient, a pH-optimum and does not depend on the presence of specific ions. There is a consumption of both serum factors. One of the factors is thermolabile and sensitive to zinc-ions. The toxin itself has subility properties different from the serum factors mentioned above. It is less sensitive to certain physical or chemical influences. The relation between anaphylatoxin formation and complement function is still to be proved.

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with the inhibition of an anaphylactic process in the skin. However, it does not appear that the process is associated with histamine-reactions. On the other hand, *we found that a certain* type of anaphylactic process leads to a decrease of the formation of anaphylatoxin in the serum.

The liberation of histamine by anaphylatoxin or by anaphylactic processes proceeds in a different manner than by low-molecular substances (for instance 48/80 or octylamin).

The inhibition of the anaphylatoxin formation by zinc-ions may be due to the affinity of the zinc to SH-groups. In this connection it is of interest to know that iodine acetate, another SH-group poison, may inhibit the liberation of histamine by conveyance of antigens to a sensitized organ *(Schild)*. Provided that the anaphylatoxin participates in the anaphylactic events, this finding may be explained by an inhibition of the anaphylatoxin formation as well as by inhibition of its effect. In fact, *Moussatché* observed that typical poisons of the SH-group are capable of inhibiting the effect of anaphylatoxin and of antigen on sensitized organs.

No unambiguous answer can be given regarding the mechanism of the histamine liberation by anaphylatoxin and the antigen-antibody reaction. The theory of ferment activation by *Rocha e Silva*, and especially by *Ungar*, is in contradistinction to some important arguments *(McIntire)*. The liberation of histamine by tryptic ferments is obviously only one of the known possibilities of this process *(Paton)*.

Now as before, the defenders of the anaphylatoxin theory of anaphylaxis face the difficulty regarding the possibility of an anaphylatoxin formation on isolated organs which can show an anaphylactic reaction. *Rocha e Silva* in this connection discusses the *formation of anaphylatoxin in intercellular fluids during the* anaphylactic process.

Perhaps further investigation will show that there is no anaphylatoxin-like humoral factor which participates in the anaphylactic shock of guinea-pigs. But the possibility remains that the process of anaphylatoxin formation in vitro is a model for what is going on in the tissues during anaphylactic shock, the tissue proteins perhaps playing the rôle of the serum proteins in vitro.

In any case, the fact that the body's own protein is able to transform into a histamine liberating substance by relatively trifling

The Gel-Precipitation Method and Its Applications in the Field of Allergy

By Ö. OUCHTERLONY, Gothenburg

(Manuscript not received)

Feinberg, J G Int Arch Allergy 11, 129—152 (1957)

Identification, Discrimination and Quantification in Ouchterlony Gel Plates

By JOSEPH G FEINBERG

From the Bencard Allergy Research Unit, Betchworth, Surrey, England

A new discovery is never as good as it first seems – and always better Let me explain. Nothing is ever as simple and straightforward as it appears in the first fresh flush of discovery. On closer acquaintance the snags begin to appear: the sensitizing *properties* of new synthetics, the resistant bacterial strains to new antibiotics the sid[illegible] grows [illegible] applic[illegible]

So it has been with *Ouchterlony*'s (1949) discovery of antigen-antibody precipitation patterns in double diffusion gel plates. What at first appeared a *simple*, straightforward method of antigen-antibody analysis, in which certain basic *rules* of "identity", "non-identity" and "partial identity" prevailed, later revealed complexities which were not simply resolved (*Kaminski*, 1954; *Jennings*, 1954, *Wilson* and *Pringle*, 1954, *Korngold*, 1956; *Feinberg*, 1957a). The idea of "one band, one antigen" was soon challenged and disproved, its converse, "one antigen, one band", has lingered longer.

On the other hand, in less than a decade widespread application has been made of *Ouchterlony*'s basic discovery in many scientific disciplines Modifications have been developed which have enhanced its usefulness.

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Author's address Prof F Hahn, M. D, Pharmacological Institute of the Medical Academy, Moorenstr 5, *Düsseldorf* (Germany)

Wells. From the start I disliked templates for forming wells in the agar. They hold up proceedings because one must wait for the gel in one plate to set before the template can be removed and used for a second plate Nor was I intrigued by the cork borer technique. With the latter, distances between wells are not accurate. So I designed, and our Instrument Shop constructed, gel cutters to meet our various needs (Fig. 1). After plates are poured and set they are lined up on the table The cutter of desired pattern is set on the first plate and pressed into the gel A drawn glass capillary tube attached to a water vacuum pump is inserted down the hollow shaft of each cutter tube and the plug removed. When the cutter is lifted out an exact pattern of clean-walled wells is left. The cutter is moved from plate to plate and the operation repeated In minutes dozens of identically patterned and spaced plates are prepared.



Fig 1 Cutters for making standardized gel plate patterns

Marking. Writing across the bottoms of the dishes is an un- [illegible] remove a reference notch from the edge of the gel and plot the antigen-antiserum layout (with reference to the notch) in the workbook. If we wish to place specific information on the gel itself, we use a solution of Alcian Blue (I C I.) and a fine artist's brush This was first described as a specific polysaccharide stain for bacterial capsules (*McKinney*, 1953). I thought it should stain agar, which is a polysaccharide, just as effectively. The Alcian Blue stains the gel indelibly and permanently, being unaffected by subsequent immersion of the gel for washing, fixing, or precipitate staining

Recording. In most cases it is desirable to make a permanent record of the precipitation patterns obtained. Simple line drawing is laborious and subject to bias and boredom – a combination deadly to accuracy. Staining, mounting and drying the gel is a slow, time-

In our laboratory we have made extensive use of the *Ouchterlony* technique for the study of antigens from pollens, microorganisms, and other sources. It is not unusual for us to set up 50 or more plates in the course of a week. Inevitably, we have become aware of shortcomings and misconceptions attached to the method, and have added some original modifications.

It is my intention this afternoon to communicate to you, within the limit of the time at my disposal, some of our observations and original developments. You may find them useful in your own work.

Methodology

It generally happens that the techniques surrounding a new method are complicated It is as though one finds it too good to believe that simple techniques could suffice to give the results required. I have never been awed by complexity, and in our work with the *Ouchterlony* plate I have made it an aim to simplify procedures as far as possible.

Agar One of the essentials in agar gel diffusion work is a clear background against which the precipitation bands can readily be seen and photographed. Several methods for obtaining clear agar have been published, but none that I saw described had simplicity to recommend it. My search for a simple and especially effective method of agar clarification was fruitful and recently I published such a method for making clean, clear agar gels (*Feinberg*, 1956). We use 1% New Zealand agar, as this gives a good firm gel. To prevent bacterial and fungal growth in the plates, we incorporate 1% sodium azide in the gel It has the added advantage of producing an ionic strength close to that of physiological saline and virtually neutral pH. We have not found buffering of the agar necessary. Adding 30 mg. of the chelating agent, ethylene-diamine-tetra-acetic acid disodium salt, eliminates artifacts due to precipitation of calcium phosphate in the gel.

Plates. Underrunning of sera and antigen solutions is another problem which has evoked some not-so-simple solutions. I find the simplest remedy is a few drops of a silicone solution, or a smear of silicone grease, rubbed into the bottom of the petri dish. It makes the glass non-wettable and effectively discourages solutions and sera in the wells from oozing under the agar

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Identification and Discrimination

I shall treat the subjects of identification and discrimination together because there is a good deal of overlapping of their provinces One must discriminate between bands even as one is attempting to identify them; and one must properly identify separate bands as belonging to more than one antigen-antibody system when one is trying to discriminate between them.

This brings up the perennial question: what exactly does a precipitation band mean in terms of antigen-antibody systems? That a single band may in reality be made up of two or more systems, whose bands are superimposed, is now well recognized. Fig 3 shows such coalescing of bands actually taking place. From it we can see that it is entirely a matter of concentration. It emphasizes the need to study the precipitation pattern at different antigenic concentrations before identifying a band as denoting a single antigenic component. Only by such study can discrimination be obtained between multiple antigens which may precipitate along the same line at certain concentrations

So much is clear and, I believe, generally recognised and accepted But there is also reason to believe that in certain circumstances one may obtain two or more bands from a single antigen-antibody system, even when one does not fill the wells more than once. I believe, in fact, that the *Liesegang* ring phenomenon is not entirely absent from the antigen-antibody reaction in gel diffusion plates I know this suggestion will arouse strong emotions, because it was a long time before gel diffusion studies could break away from *Bechold's* (1905) original misconception that all multiple bands were due to the *Liesegang* phenomenon, rather than to a multiplicity of antigens and antibodies It is not my intention to revert to the notions of *Bechold*, but solely to point out that sometimes the *Liesegang* phenomenon may slip into our plates to confound our attempts at identification and discrimination.

Liesegang's rings, if I may recall briefly, are noted particularly in inorganic systems. When silver nitrate, for example, is allowed to diffuse through a tube of gelatine containing ammonium chromate a rhythmic precipitation of silver chromate takes place if the salt concentrations are right – i.e. a series of precipitation bands forms in the gel *Hughes* (1934), explaining the *Liesegang* phenomenon, states that the nature and degree of banding depends on the

consuming process. We decided photography offered the greatest promise for routine, accurate recording of precipitation patterns. Available photographic methods fell short of the results we sought. Fortunately, our staff photographer took a keen interest in the problem and, with the aid of the Instrument Section, an apparatus was constructed (*Lawson*, 1957) which produced the better of the photographs you will be seeing today.

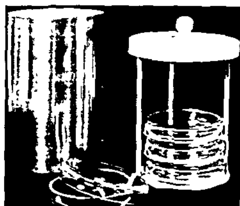


Fig. 2 Cannister for gel plate storage

Storage. Prevention of evaporation is essential while the precipitation bands are forming. Resort to large desiccators or other air-tight devices is unnecessary. A tin with a loosely fitting lid suffices, if one places a beaker of water in the tin with the plates. For incubation of plates at 37° C we use a modified bacteriological petri dish cannister (Fig. 2), in which the carrier is somewhat shorter than the cannister itself and a little water is kept on the bottom of the latter. Individual dishes can be incubated or stored indefinitely with a layer of white oil over the gel, but this can become messy in handling.

Once the precipitation pattern is obtained, I have found it can be fixed and kept from distorting by layering the gel with an approximately equal volume of 20% methanol in physiological saline. This also makes the bands stronger. It is often invaluable for showing up the bands formed in weak systems. Alternatively, stained gels can be dried on siliconed glass plates and the agar films removed from the glass and filed.

But if we look further we can see a form of banding which I think is independent of temperature changes. Between wells 2 and 1 (reading clockwise) we find a band spreading out and becoming two. It is the outermost band of well 2 where it most closely approaches well 1. A possible explanation is that the precipitation around well 1 has drawn on antibody from the interzone between the two wells, giving rise to an area of antibody "rarefaction". Therefore, the antigen diffusing from well 2 has been able to travel farther afield in this area and reprecipitate along the bulge. In the case of temperature changes, similar areas of antibody "rarefaction" – or, more orthodoxly, antigen excess – may be created because lowering of the temperature may slow the diffusion of the large gamma globulin antibody molecules more than the smaller ovalbumin molecules.

Fig. 5 represents a different case. This is a conventional *Ouchterlony* plate in which the antiserum was in the centre cup and the antigen in the three peripheral cups. I think you can see the intense bands formed within the broad band. I think that in this plate our relative concentrations were such as to produce "close bands with 'spaces' filled with precipitate, the bands being only distinct through being more intense and compact".

But how can antigen or antibody break through the barrier of a precipitation band to form additional bands? We already recognise that a precipitation band is no barrier to the passage of [illegible] no actual [illegible] a photomicrograph of a precipitation band in an agar gel (Fig. 6). It is apparent that what may appear as a solid band to the naked eye is in reality a collection of discrete precipitate particles I think it reasonable that, in view of what we saw before, the spaces between the particles are "rarefied" in respect of antigen and antibody molecules So it is entirely possible that such molecules may pass through the band zone without meeting their opposite numbers; and so be available, if conditions beyond are right, for precipitation in a second zone This agrees with the views of *Jennings* (1954) and *Jennings* and *Malone* (1954) If I may borrow an analogy from nuclear physics, the mean penetration distance of a neutron is only a statistical average, which may be well exceeded by any individual neutron Likewise, the zone in which a precipitate forms in a gel

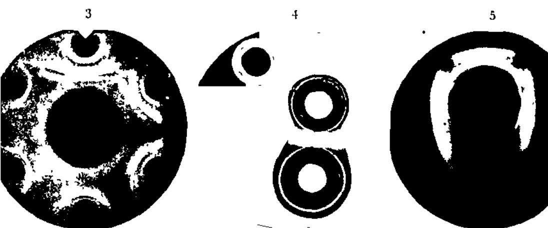


Fig. 3 Hazel pollen extract Double serial clockwise from well 1 (notch) Note coalescing of bands with decreasing antigen concentration (wells 3–6)

Fig. 4 Single antigen-antibody system producing multiple bands Possible Liesegang ring formation

Fig. 5 Liesegang ring-like phenomenon within zone of precipitation of single antigen-antibody system

relative concentrations of the two reactants, and that it can range from a continuous precipitate without discernible bands, to a series of widely spaced clearly separated bands. Any intermediate type of banding may also be procured: such as rather close bands with fairly clear spaces; or close bands with "spaces" filled with precipitate, the bands being only distinct through being more compact and intense.

Figs. 4 and 5 demonstrate what I consider to be two levels of *Liesegang* band (or ring) formation in a single antigen-antibody system. The system used is a crystallised ovalbumin, prepared and four times recrystallised in our laboratory, and an antiserum to it made in a rabbit. *By no conventional method of Ouchterlony plate analysis have we been able to demonstrate more than one antigen or antibody in this system.* Yet, if we place a solution of the ovalbumin in a well in a gel which has the antiserum incorporated in it we can, when concentrations are right, produce a series of concentric rings (Fig. 4) merely by alternately warming the plate to 37° C and cooling it to room temperature. I think this is a case of temperature-conditioned *Liesegang* rings in which there are "rather close bands with fairly clear spaces". The explanation may be that during the periods of cooling the larger antibody gamma globulin molecules are slowed down more than the smaller ovalbumin molecules, causing a shift in the zone of equilibrium.

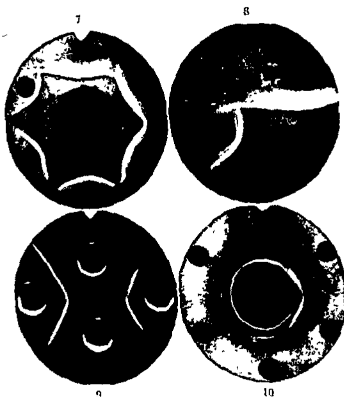


Fig 7 Crossing-over ("reaction of non-identity") of identical precipitation bands (between wells 6 and 1)

Fig 8 Enlargement of crossing-over area from Fig 7

Fig 9 Spur formation ("reaction of partial identity") between cross-related antigens. native ovalbumin and chemically substituted ovalbumin

Fig 10 Gel plate precipitation of a simple chemical substance (Double serial dilution clockwise from well 1)

which a spur is formed at the point where two adjacent bands join up (Fig 9) From what I have already shown it is obvious that spur formation need not necessarily represent partial identity. If two bands are superimposed and one of those bands is identical with that at an adjacent well, a linkage would take place between the identical bands, yet leaving the dissimilar component of the compound band to go off as an apparent spur. Conversely, the reaction of partial identity may fail to occur despite the true partial identity of two adjacent antigens.

For illustration I turn to a study which arose from an interest I had in the possibility of employing a simple synthetic chemical for

may only represent the *statistical average path* travelled by the antigen and antibody molecules before being stopped by precipitation.

Now I must take exception to the so-called "reaction of non-identity". When two approaching bands cross each other without joining up, it is taken to imply that the antigen-antibody system of one is different from that of the other. As far as I know this principle

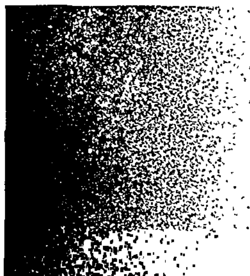


Fig 6 Photomicrograph of precipitation band in agar gel

has so far gone unchallenged But in Fig. 7 we have evidence that such is not always the case. We have there six double serial dilutions of our crystallised ovalbumin in the peripheral wells, with the antiserum in the centre well There can be no question about the solution in well 6 being different from the solution in adjacent well 1 in any respect other than concentration. Yet you can clearly see the bands do not join up. *They cross each other*. The crossing over is even more clear in this enlargement of the critical area (Fig. 8). One special condition exists in this plate: the serum was allowed to diffuse 24 hours before the antigen was added. But that is immaterial to the conclusion to be drawn: that when relative concentrations of antiserum and antigens are right, it is possible to have a "reaction of non-identity" between identical antigen-antibody precipitation bands.

It remains for me now but to challenge the "reaction of partial identity" and I shall have tilted at the lot. The reaction is one in

molecule. Thus it meets its equivalence of antibody much closer to the serum well. The band it forms is then so far from that formed by the coupled ovalbumin that it is physically impossible for it to demonstrate its partial specificity with the latter by hooking up with it.

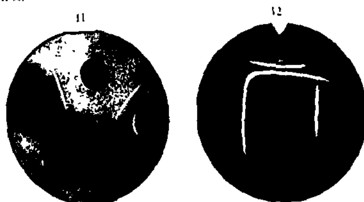


Fig 11 Failure of spur formation [illegible]
tion bands T[illegible]
hapten, right [illegible]

Fig 12 Intragel specific absorption Mixed antigen-antibody systems, unabsorbed (see text for details).

I have spent some time crying "Beware"! I feel I was justified in so doing because before we can make proper use of a method we should understand its mechanics and be aware of its limitations. In the case of the *Ouchterlony* plate the nature of the molecules we are deal[illegible] [illegible] taken into account it is all too easy to draw false conclusions from the precipitation patterns observed. With that warning, let me get on to the brighter side of my paper.

This will concern itself with identification and discrimination in complex antigen systems All of you who have studied allergenic extracts from natural sources in gel plates will know but too well the antigenic miscellany such extracts contain In attempting to compare them with other related extracts – such as those from different pollens – it is virtually impossible to follow accurately the joinings, partial joinings and crossings-over of the individual antigen bands.

the purpose of clarifying some of the phenomena involved in *Ouchterlony* gel plates. The chemical chosen for the work was one of *Pauling*'s (1942) synthetic divalent haptens: dihydroxy-di-(p-azo-phenylarsonic acid)-benzene, a molecule in which two prosthetic arsanilic acid groups are attached to a resorcinol nucleus. When arsanilic acid is diazotized and itself coupled to our ovalbumin and the coupled ovalbumin used for immunisation, one gets an anti-serum which is in part specific for the arsanilic acid moiety.

In theory one should be able to get a precipitation band in a gel between this antiserum and the divalent resorcinol-arsanilic acid. The theory worked in practice (*Feinberg* and *Grant*, 1957). A band was formed between the antiserum and varying concentrations of the hapten (Fig. 10).

When native ovalbumin and coupled ovalbumin are run against their homospecific and heterospecific antisera we get the pattern seen in Fig. 9. Between the anti-native ovalbumin serum and the two antigens we get but a single continuous band, formed by the joining of each of the two bands. This is what we would expect, since the antiserum is unispecific for ovalbumin and the ovalbumin has evidently not entirely lost its native specificity when coupled with the arsanilic acid. On the other side, we find a spur being formed where the precipitates formed against the anti-coupled ovalbumin serum join together. This again would not be unexpected, since this antiserum is bispecific – i.e. it has specificities both for native ovalbumin and the arsanilic group. The bands it forms against the two antigens have a partial identity – to native ovalbumin – so they join. But in addition the antiserum has its second specificity to the arsanilic group of the coupled ovalbumin – and this manifests itself as a spur of precipitate which declares its independence from the native ovalbumin band by going off at a tangent.

This is a classic concurrence with theory. But now for the exception to theory (Fig. 11). Here again we have two antigens with partial identity. In the one cup we have placed the divalent hapten, in the other the coupled ovalbumin You will see that each has formed its own entirely independent band, despite its partial identity with the other. The reason, I believe, is apparent Owing to the very much smaller size of the synthetic antigen it races through the gel much more quickly than the more cumbersome ovalbumin

molecule. Thus it meets its equivalence of antibody much closer to the serum well. The band it forms is then so far from that formed by the coupled ovalbumin that it is physically impossible for it to demonstrate its partial specificity with the latter by hooking up with it.

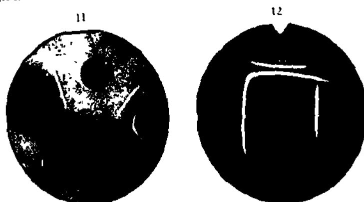


Fig 11 Failure of spur formation between partially related antigen-antibody precipitation bands Top well- chemically coupled ovalbumin, bottom well *chemical divalent* hapten, right well anti-coupled ovalbumin serum (Hapten band slightly retouched for photography).

Fig 12 Intragel specific absorption Mixed antigen-antibody systems, unabsorbed (see text for details)

I have spent some time crying "Beware"! I feel I was justified in so doing because before we can make proper use of a method we *should* understand its mechanics and be aware of its limitations. In the case of the *Ouchterlony* plate the *nature* of the molecules we are dealing with, and their absolute and relative concentrations, are critical Unless these factors are known, controlled, or otherwise taken into *account it is all* too easy to draw false conclusions from the precipitation patterns observed. With that warning, let me get on to the brighter side of my paper

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Some time back *Björklund* (1953) published several excellent papers on a method for reducing the number of antigen-antibody bands, thus simplifying the systems under study, by adding antigen to the agar gel. Unfortunately, this work has not attracted the attention it merits. *Wodehouse* (1956), in his "Quasi-critical review of gel diffusion", does not refer to it in his section on techniques. And from the number of papers published in which no advantage has been taken of the band reduction technique it is obvious that many workers are unacquainted with it.

Recently (*Feinberg*, 1957), I undertook a detailed, definitive study of this technique, which I term "intragel specific absorption" for, in effect, it is analogous to the absorption technique of classical tube precipitation. Because I feel the potentials of the method in the discrimination and identification of antigens and antibodies makes it worthy of wider attention, and since the work I carried out is in some respects a critical analysis of these potentials, I will describe the principle of intragel specific absorption here.

For the purpose of my study I created an arbitrary system involving antigens from two distinct sources and a mixture of the antisera to the two (Fig. 12). The mixed antisera went into the central well. In the well on the right is a commercial bovine fibrinogen preparation. You will see that it is heterogeneous and we have been able to demonstrate as many as four distinct antigens in it. In the left well I have placed that workhorse of our gel diffusion studies: our crystallised ovalbumin. You will note again its apparent antigenic purity. In the top well is a mixture of the two. We see each of the antigens showing its presence independently and joining up with its fellow in the adjacent cup where physically possible However, the innermost fibrinogen line has disappeared in the mixed antigen system and we are left to conjecture whether it is simply obliterated by the overlying, stronger ovalbumin band, or whether the latter has actually interfered with its formation.

We resolve the problem simply when we resort to intragel specific absorption and incorporate ovalbumin in the gel (Fig. 13) As the mixed antibodies begin to diffuse from the central well the anti-ovalbumin immediately meets up with the intragel ovalbumin, is absorbed by it and precipitated out It is visible as a ring of precipitate just around the antiserum well This eliminates the albumin bands and the full complement of precipitate bands for the fibrino-

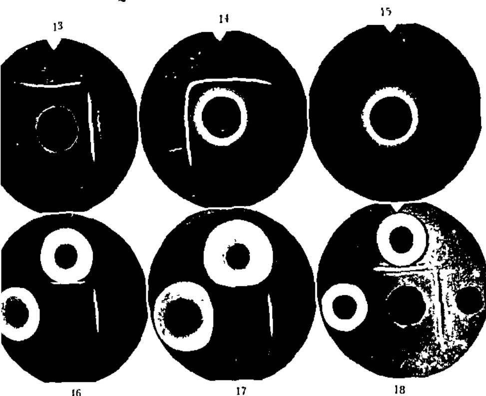


Fig 13 Intragel specific absorption Purified ovalbumin in gel.
Fig 14 Intragel specific absorption Commercial fibrinogen in gel
Fig 15 Intragel specific absorption Ovalbumin and fibrinogen in gel
Fig 16 Intragel specific absorption Anti-ovalbumin in gel (5%) Note masking of outermost fibrinogen band
Fig 17 Intragel specific absorption Anti-ovalbumin (2 5%) in gel Note unmasking of outer fibrinogen band
Fig 18 Intragel specific absorption Anti-ovalbumin (10%) in gel Note unmasking of outer fibrinogen band

gen preparation appears. An antigen is unmasked which would otherwise have gone unseen.

In Figs 14–20 we see the converse, in which the fibrinogen bands are eliminated by intragel absorption with the commercial fibrinogen, elimination of all bands by absorbing in the gel with both antigens, and that intragel specific absorption of antigens can be accomplished by incorporating antiserum in the gel, with a similar specific elimination of precipitation bands. In Fig 17 we also see the heterospecific band of one antigen actually formed

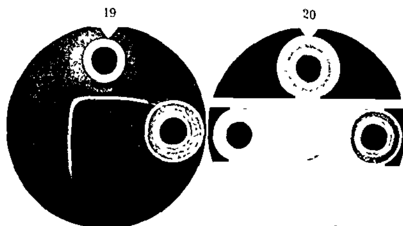


Fig. 19 Intragel specific absorption Anti-fibrinogen in gel
Fig. 20 Intragel specific absorption Anti-ovalbumin and anti-fibrinogen in gel

within the zone of precipitation of another, proving that unrelated antigen-antibody systems act quite independently of each other.

You will recall the pattern of partial identity we got in our ovalbumin and arsanilic-coupled ovalbumin plate (Fig. 9) We surmised at the time that the spur was due to the dual specificity of the coupled ovalbumin. Intragel absorption provides us with the proof (Fig. 21). When native ovalbumin is added to the gel all the ovalbumin bands disappear, but between the coupled ovalbumin and its antiserum a strong band persists, running off in the direction of the spur on the unabsorbed plate. This shows the spur was due to a second specificity on the coupled ovalbumin molecule: a specificity due to the attached arsanilic group. Absorb with coupled ovalbumin in the gel and all the bands disappear (Fig. 22). The coupled ovalbumin absorbs completely, as it carries the two specificities involved in the antigen-antibody system.

Such, then, are the potentials of intragel absorption in helping us to identify and discriminate between precipitation bands in *Ouchterlony* plates An obvious application of the technique is in the study of the components of complex antigen-antibody systems, especially between systems which have some components in common. Absorption by the elements of one system, by eliminating bands attributable to common components, will demonstrate its relationship to other systems. *Such investigations are now under way* in our laboratory on pollen extracts, particularly with a view to elucidating the recognized, but so far poorly defined, relationship between the pollens of the grasses. And as we have seen so clearly

demonstrated on one of the slides I have shown, in our hands intragel absorption has already made clear one point about which there has in the past been some dispute. It has shown that there is no obvious effect on the pattern, placement or intensity of the bands of one precipitating system by the diffusing antigens and antibodies of a second system. In fact, even in a zone of antigen-antibody reaction there is no interference with the bands of hetero-specific precipitates.

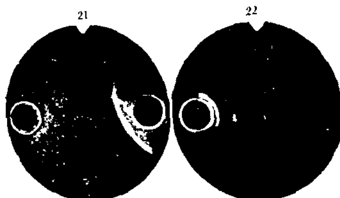


Fig 21 Intragel specific absorption in cross-related systems Elimination of one antigenic specificity

Fig 22 Intragel specific absorption in cross-related systems Elimination of both antigenic specificities (Band at left well is an artefact)

Quantification

This brings me to the final section of my talk: quantification in *Ouchterlony* plates This is a very new field. When *Wodehouse's* review appeared in 1956 it stated "With *Ouchterlony* plates quantitative determinations are not possible". Shortly thereafter I published a preliminary description of a method for such quantitative determination (*Feinberg*, 1956)

We were led to our search for a good routine method for quantitative antigen analysis because of our dissatisfaction with the available methods for the assay of allergens Both the Noon unit and the protein nitrogen unit are in themselves meaningless The one ignores the efficiency or lack of efficiency of the extraction process, the other ignores the possible allergenic role of polysaccharide and turns a blind eye to the indignities the protein may have suffered in the course of the extraction The highest protein

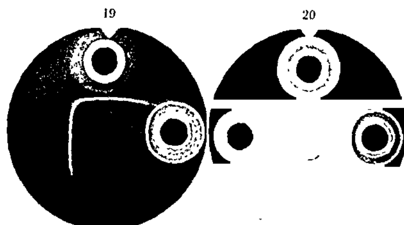


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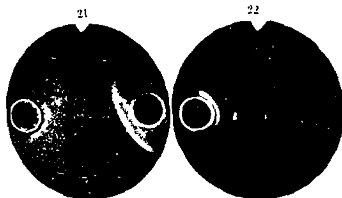


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We were led to our search for a good routine method for quantitative antigen analysis because of our dissatisfaction with the available methods for the assay of allergens. Both the Noon unit and the protein nitrogen unit are in themselves meaningless. The one ignores the efficiency or lack of efficiency of the extraction process; the other ignores the possible allergenic role of polysaccharide and turns a blind eye to the indignities the protein may have suffered in the course of the extraction. The highest protein

nitrogen level we ever reached in any of our experimental pollen extracts was in a hot 0.1 M NaOH extract. Needless to say all the immunological specifity of the protein had been destroyed.

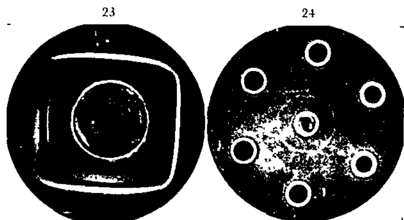


Fig 23 Double serial dilutions of a single pollen extract, showing fallacy of "weak" and "strong" bands (clockwise from top well)
Fig 24 Serial Dilutions of pollen extract 100 (top well), 80, 64, 51, 41, 33 Note insensitivity of this type plate

The only sort of assay which can have any semblance of meaning is one which would measure the specific antigenic activity of an extract, particularly if it also made possible the individual assay of each of the antigens in a heterogeneous mixture. If, at the same time, the method could give a qualitative picture of a heterogeneous mixture – and in allergy one is inevitably dealing with heterogeneous mixtures – it would come close to approaching an ideal. It was almost too much to hope that such a method, if it could be found, would also be simple, but it was worth searching for. Only a method based on the *Ouchterlony* plate seemed to me to offer any prospect of meeting these conditions.

I am glad to be able to report my faith in the *Ouchterlony* plate was vindicated. With it I was able to perfect a quantitative assay method with all the desired attributes – plus a sensitivity which made possible reproducible results on the 10–20% level, by immunological standards a high degree of accuracy.

Fig. 23 demonstrates the fallacy of attempting to draw quantitative conclusions from qualitative gel plates and the deceptiveness of judgements based on "weak" and "strong" bands, terms which crop up constantly in the literature. In this figure we have four

double serial dilutions of a timothy pollen polysaccharide preparation The preparation contains, as you will see, several components. Bear in mind that these components were diluted equally, as they were all in the same original solution. Yet note what happens. The outer component gives a "strong" band at the highest concentration and a "weak" band at the lowest concentration. This is as one would expect. However, the inner components start off extremely faint in the strongest concentration and do not become a "strong" band until we get to the lowest concentration, passing through a 3-band stage on the way.



Fig 25 Cutter for quantitative gel plates

This illustrates forcibly the impossibility of making a quantitative judgement from this type of plate. Had we attempted to pass judgement on the basis of a single test with one concentration of antigen we could have arrived at two diametrically opposite conclusions, depending on whether we had chosen the concentration in the first well or that in the last. In the first instance we should have concluded that there was a strong outermost component with virtually little else. In the second we would have concluded that there was a very strong inner component and a weaker outer one.

The starting point of my search for a quantitative method was the conventional *Ouchterlony* plate. We see in Fig 24 what happened when I attempted to titrate a series of dilutions of a goldenrod pollen extract. It was most discouraging, for one could see little difference between the first dilution and the last, which was only one-third as strong On theoretical grounds I redesigned the gel pattern to be studied and designed a new style of cutter (Fig. 25).

It is a quite simple device, made in our own Instrument Shop to precision standards The distances between the cutting tubes are accurate – as measured by a travelling microscope – to plus or minus 1%. It is sturdy and virtually indestructible The one you

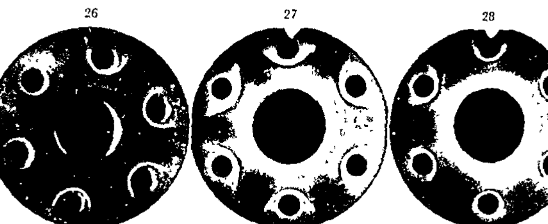


Fig. 26 Serial dilutions of pollen extract: 100 (top well), 83, 71, 62, 55, 50 Note ring endpoint at well 5

Fig 27 Quantitative comparison of two antigens Wells 1–3: serial dilutions of antigen A; wells 6–4 serial dilutions of antigen B

Fig 28 Quantitative comparison of two antigens. Extension of dilutions from plate Fig 27

see pictured here has been used in our own Laboratory for a year and is as sharp and as accurate as the day it was made. Having once gone to the trouble of getting the instrument made our difficulties were thereafter ended. With it, with a minimum of effort and no tedious measurements of distances, we can turn out an endless line of quantitative agar plates which are exact replicas of each other from day to day and from year to year.

Fig. 26 shows what this cutter is capable of accomplishing. It is a titration of a goldenrod pollen extract in which we have succeeded in differentiating between concentrations at the 10% level. The endpoint is the first well which is completely encircled by a ring of precipitate

In alternative methods of setting up the plates a direct comparison can be made on *the same plate between two or more* antigens, or between unknown and standard antigens. In the first variation (Figs. 27 and 28) the two antigens under comparison (in this case two different extracts of cocksfoot pollen) are made up in the same serial dilutions. The first three dilutions of each are placed in one plate, another three in a second plate. This could, of course, be carried through any number of plates. Note that an endpoint is reached in this lowest dilution of the one antigen, whereas the other has not yet reached its endpoint. It would have been better to have continued into a third plate, but even in the absence of the latter

. . . .rengths antigen his case indicating the weaker is two-thirds the strength of the stronger. In a second variation (Figs. 29 and 30), using our ovalbumin, one antigen is kept at constant dilution and placed in alternate cups, while the second is diluted serially and placed in the cups between. It is then a simple matter to match the respective precipitation bands This variation is, in effect, a comparator method for measuring antigenic potencies.

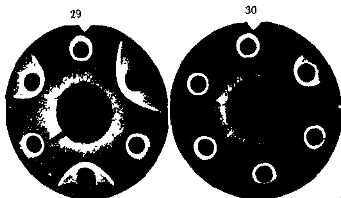


Fig 29 Quantitative antigenic comparator plate Wells 1, 3, 5 antigen A in single concentration Wells 2, 4, 6 serial dilutions of antigen B

Fig 30 Quantitative antigenic comparator plate Extension of plate Fig 29

Fig 31 demonstrates the reproducibility of the method. It shows two duplicate sets of plates, each set comprising two plates with 12 serial dilutions of ovalbumin. Note the continuity achieved between the two cups of each set, and the identical patterns obtained in the corresponding cups of the two sets With an "Agla" syringe I have determined that even in a thin gel one routinely fills the wells, which hold 0 03 to 0 04 ml , with an accuracy of plus or minus 3 % Incidentally, this slide also shows the sensitiveness of the method The faint ring of precipitate you can see round the last well is made by ca 0 05 μg of ovalbumin.

There are two additional outstanding advantages of this method over quantitative gel diffusion methods in tubes the superb qualitative picture one obtains of the antigenic pattern of a complex antigenic mixture, and the ability to titrate separately each of the

individual antigens (Fig. 32). In this plate, showing a series of dilutions of a hazel pollen extract, successive endpoints are achieved for each of three different antigens, while it is also possible to study the dilution pattern of those antigens which do not reach positive endpoints on the plate. How much richer in qualitative and quantitative information this quantitative plate method is than the quantitative tube methods.



Fig 31 Two identical sets of plates showing extensibility and reproducibility of method

We have thus seen the capabilities of this new quantitative method for assaying antigens in agar gel plates We have seen that not only is it very sensitive, but also sensitively discriminating. It allows us to assay in a complex mixture of antigens each of the component antigens, one by one. To those who work with allergenic extracts, those complex antigenic pot pourris whose qualitative and quantitative compositions may change from extract to extract and season to season, any in vitro assay method which offers less is but a blunderbuss approach, not far superior to the protein nitrogen method of assay.

However, there are times when and situations in which less critical analysis will suffice While working at my refined quantitative technique it became obvious that with slight modification it could be transformed into a somewhat simpler method which would suffice for such less demanding situations. This was but a quantitative refinement of the work of *Petrie* (1932). Instead of establishing a serum gradient in the gel, the serum is actually incorporated in the gel at a suitable concentration – generally 5 or 10%. Serial dilutions of the antigen are placed in wells cut in the agar. With a cutter I designed it is a simple matter to set up any number of plates with identical patterns of 18 wells, all of identical size (Fig. 33) One can, as I have done on this plate, use three sets of six holes to titrate simultaneously on the one plate three different preparations.

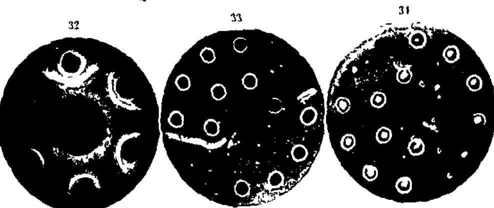


Fig 32 Titration of individual antigens of heterogeneous system on one plate (wells 1, 2, 6).
Fig 33 Antigen titration in triplicate, after one-half hour.
Fig 34 As Fig 33, after 24 hours

Actually in this plate I have used the same solution three times to demonstrate the reproducibility of the method. It is seen that the last ring of precipitate forms in each case around well 4. That endpoint was reached in one-half hour at 37° C, this being the photograph of the plate at that time. At 24 hours (Fig. 34) the endpoint is unchanged This method offers, then, a quick method for total antigen assay, but one which is neither as sensitive nor discriminating as the serum gradient method.

The white blobs inside some of the wells are actually specific precipitates formed by diffusion of the serum from the agar into the antigen-containing wells. The conditions present – i.e. fixed antiserum concentration in the presence of varying antigen concentrations – and the manner in which the precipitates formed were reminiscent of the classical Dean and Webb optimal flocculation system Would it be possible to assay various antigens in terms of a given antiserum by observing their optimal flocculation concentrations in gel plates? Experimentation showed this was indeed a practical possibility.

In fact, this plate was set up to study just that phenomenon. In Fig 33 one can just see evidence of flocculation taking place, principally in the fourth well After one hour incubation at 37° C (Fig. 35) the flocculation is more easily photographed, and it is clearly seen that in each case it has been maximum in the fourth well This offers an interesting alternative to the previous method

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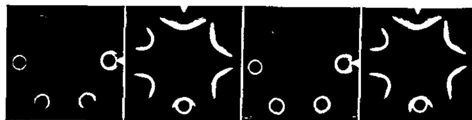


Fig. 31. Two identical sets of plates showing extensibility and reproducibility of method

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used the *Ouchterlony* gel plate is an extremely flexible tool which can be modified to tackle a host of immunological tasks. I hope that both the Jeremiah and Zechariah characters of my talk may find some application in the work you may yourselves be doing on antigen-antibody analysis.

Finally, I should like to thank those of my colleagues who have in one manner or another contributed to the material around which my talk was built. Dr. *R. A. Grant* was responsible for the synthesis of the divalent hapten and the coupled ovalbumin. Miss *Hazel Grayson* prepared the recrystallised ovalbumin. Both have set up many gel diffusion plates. Mr. *D. F. Lawson* tirelessly worked to perfect the photographic technique which made possible the photographs he prepared to illustrate this paper, while Mr. *R. D. Peters* made the gel cutters And to Dr. *F. H. Milner*, Technical Director of C. L Bencard Ltd, and to Dr. *J. Farquharson*, Director of Beecham Research Laboratories, I am indebted for encouragement in my work and for permission to present it today at this distinguished meeting.

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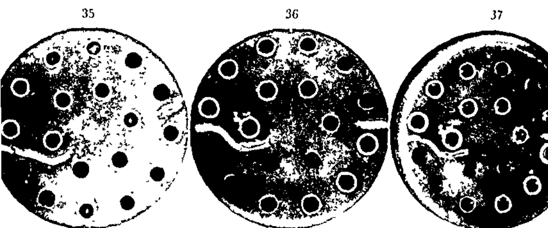


Fig 35. Antigen-antibody precipitation in wells
Fig 36 Anti-serum titration in triplicate, after one half hour
Fig. 37 Anti-serum titration in triplicate, after 24 hours

for gross antigen titration in antiserum-agar. We have not yet had the opportunity to compare the relative accuracies and sensitivities of the two methods, but intend to do so in the near future.

There is yet one more modification I have made in the *Ouchterlony* plate to adapt it to quantitative immunological assay This is in effect the reverse of the previous modification, i.e. an antigen-agar is used for the assay of antisera (Fig. 36) The pattern is the same as in the previous plate, but this time the antigen is in the agar and serial dilutions of the antiserum in the wells. Again an endpoint develops within one-half hour at 37° C and remains unaltered after 24 hours (Fig. 37) For the routine assay of antisera this is a convenient and reasonably accurate technique, especially when used as a battery method such as with the 18-hole cutter.

In conclusion, I should like to pay tribute to Prof *Ouchterlony*, whose original discovery of gel diffusion studies in plates has given us such a powerful tool for immunological analysis. I hope that I have been able to make some small contribution toward widening the scope of the *Ouchterlony* plate in our work with allergenic substances. If I started out a Jeremiah, casting gloom about the very temple of the *Ouchterlony* plate, I trust I have finished up a Zechariah predicting for it a new and bright future as a unique foundation for immunological quantification. But even gloomy prophecies have their use, and in the present case I hope they may serve to foster caution in the interpretation of precipitation patterns obtained in gel plates. On the brighter side, I think I have shown that properly

Augustin, R. Int. Arch. Allergy 11 153—169 (1957)

Fundamental Aspects of Single Versus Double Diffusion Methods for Immunological Assays

By R. AUGUSTIN

From the Wright-Fleming Institute, St Mary's Hospital Medical School, London, W. 2

Oudin's brilliant realisation of an immunochemical analysis of complex antigen-antibody systems without the laborious isolation of the reactants has been developed in numerous ways, by himself and other workers. In the present paper only quantitative aspects will be considered.

1. *Single Diffusion*

Antigen as the external reactant

In the original experiments ("diffusion simple") *Oudin* overlayered a column of agar containing a constant concentration of antibody (Ab), called the internal reactant, with a solution of antigen (Ag), called the external reactant. At sufficiently high Ag to Ab concentration ratios Ag diffuses into the gel layer and reacts there with the Ab. Ag-Ab precipitation zones will start to form at the liquid-gel interface and will then be seen to stretch further and further down the tube as more Ag eats its way into the Ab layer, clearing it of free Ab in the process. In precipitin ("rabbit") systems (Ag-Ab precipitate soluble in excess Ag and insoluble in excess Ab, see fig. 1) the leading edge is well defined, but the trailing edge may become ragged, and may finally dissolve in its upper parts where there is a large excess of Ag. At what distance from the leading edge this occurs depends upon the ease and rapidity with which the particular Ag-Ab precipitate dissolves in excess Ag. In flocculating ("horse") systems (narrow range of precipitation, precipitate soluble in excess Ab as well as in excess Ag, see fig. 1) lines rather than long drawn out zones of precipitation can be

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Keeping the Ab concentration constant, and using various Ag concentrations C, *Oudin* found empirically that a straight line is obtained when $\frac{x}{\sqrt{t}}$ is plotted against log C, i. e.

$$\frac{x}{\sqrt{t}} = A \log C - B \qquad \ldots(2)$$

Where A and B are constants.

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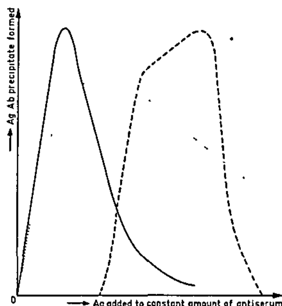


Fig. 1. Principal types of Ag-Ab precipitation curves Increasing amounts of Ag are added to constant amounts of Ab. ———— 'rabbit' precipitin type. Ag-Ab precipitate soluble in excess Ag and insoluble in excess Ab – – – – – 'horse' flocculation type: Ag-Ab precipitate soluble in excess Ag and excess Ab ...... carbohydrate precipitin type: Ag–Ab precipitate insoluble in excess Ab (horse or rabbit) and not very soluble in excess Ag

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According to the usual laws of diffusion the distance x of the leading edge from the interface at any time t would then be found to be proportional to $\sqrt{t}$ Thus we may write

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Where k is a constant whose value will depend upon the substances used and their concentrations.

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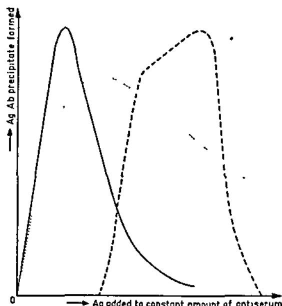


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A more rigorous mathematical treatment (*Spiers* and *Augustin*, 1957) is available, in which Ag-Ab interaction – amounting to mutual "annihilation" in the reaction zone – is taken into account. The results agree roughly with the deductions of *Becker* et al. with regard to small enough or large enough ratios of Ag-Ab concentrations. In addition, the value of $k = \frac{x}{\sqrt{t}}$ for any ratio of concentrations can also be predicted in terms of known quantities, and is given by

$$\frac{[1 + \mathrm{erf}\,(k/2\sqrt{D_1})]\ \exp\,(-k^2/4D_2)}{[1 - \mathrm{erf}\,(k/2\sqrt{D_2})]\ \exp\,(-k^2/4D_1)} = \frac{C_1}{C_2}\sqrt{\frac{D_1}{D_2}} \quad \ldots(6)$$

where D_1 and D_2 are the diffusion coefficients, and C_1 and C_2 the initial concentrations (in 'equivalent' units) of the Ag and Ab respectively; 'exp' denotes the exponential function, and 'erf' the normal probability integral or 'error function', both of which are tabulated in most books of mathematical functions. Comparison with experimental data has proved satisfactory for the system bovine serum albumin (BSa)-rabbit antibody (*Augustin*, *Hayward* and *Spiers*, to be published) over a wide range of concentration ratios (from 1 to 4000).

Determination of Ab-concentrations (and equivalence ratios)

In general the *Oudin* method has not been used for the estimation of Ab-concentrations although there are in principle four ways in which this can be done.

1. a) Two Ab-concentrations may be compared by incorporating them into a gel *(Ag as the external reactant)* and determining which Ag concentrations give equal depths of penetration in both instances. Theoretically, the ratio of these two Ag-concentrations should then be the same as that of the two Ab-concentrations.

1. b) The concentration of one of the internal reactants (Ab) may be varied while keeping the Ag-concentrations *(Ag as the external reactant)* constant. As before equal depths of penetration should indicate equivalent Ag-Ab ratios, i.e. one Ab-concentration may be expressed in terms of another.

Oudin (1952) claims to have obtained straight lines by plotting $\left[\frac{x}{\sqrt{t}}\right]$ against the logarithm of the Ab-concentrations. Indeed, by exterpolating these straight lines to zero movement ($x = 0$ for all t) he claims to be able to obtain rough estimates for the equivalence ratio R – "except in extreme cases". From his fig. 8 (*Oudin* 1952) equivalence ratios of 20, 18 and 20 may be deduced for absolute Ag-concentrations (presumably in arbitrary units) of 10, 100 and 1000 respectively. Yet for Ag-concentrations of 1 and 10 000 units the corresponding values of R are 50 and 63 respectively according to this same figure. While it is clear from other work (*Becker* et al., 1951, *Spiers* and *Augustin*, 1957) that *Oudin*'s equations can only hold good for a very limited range of Ag-Ab ratios, it is a little disturbing that it should matter what absolute concentrations are used. At high concentrations some interference with diffusion processes may be conceded on general grounds, but deviations at low concentrations remain unexplained.

2. *Ab* may be used *as the external reactant*, in which case the Ab-concentration must exceed that of the Ag. In principle it should again be possible to make comparisons of Ab concentrations by varying either the Ab-concentration at constant Ag concentration (2a) or by varying the concentration of the internal reactant (which is the Ag this time) while keeping the Ab concentration constant (2 b).

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Ag-Ab interface without passing anywhere through a maximum. However this is *a priori* not a reason for not using such a reversed test for quantitative purposes in pure Ag-Ab systems; *Oudin*'s own microphotometric tracing (*Oudin*, 1952) shows that the leading edge of precipitation is clearly defined. Increasing density behind the leading edge (with Ab as the external reactant) would seem to be the exact counterpart of the decreasing density (dissolution) behind the leading edge where Ag is the external reactant. In both instances complexes of a higher ratio of external to internal reactant are found in the trailing edge than in the leading edge. The existence of these secondary complexes in the trailing edge is neglected in all the mathematical treatments, i.e. deviations from the assumed proportionalities may be expected, depending on the rate of formation and the composition of these secondary complexes, but *not* on their visibility.

There is, however, a very important difference; a given quantity of Ag (Ag loaded gel) can bind a much larger amount of Ab (multivalency of Ag) than the amount of Ag (Ab loaded gel) that can be bound by the equivalent amount of Ab (bivalency of Ab).

When using immuno-electrophoretically pure crystalline bovine serum albumin (BSa) as the internal reactant and its rabbit antiglobulin as the external reactant we obtained two very slowly advancing leading edges; the upper one, very slow, pertained to an extremely dense (but evenly dense by naked eye observation) zone of precipitation, while the lower one moved at about double the rate of the upper one and belonged to a much less dense zone of precipitation. At least two explanations are possible for this unexpected appearance of two zones of precipitation. (1) Rates of forming complexes of a high Ag-Ab ratio may be distinctly slower than rates for forming complexes of the equivalence ratio. However, the very sharp onset of the second zone of precipitation makes it more probable that (2) the BSa contained a very small amount of impurity, and the rabbit antiserum a disproportionately large amount of Ab to this impurity – as has been known to occur in other systems (*Wetter* and *Deutsch*, 1952). This would result in a very high Ab-Ag ratio for the impurity and a low Ab/Ag ratio for BSa itself; the dense slowly moving upper zone would thus represent the BSa system, and the much less dense and more quickly advancing lower zone would be due

to the impurity. Even a large amount of Ab to an Ag impurity would not interfere when Ab is the internal reactant. On the other hand a suitably controlled reversed *Oudin*-method could serve as a very sensitive tool for discovering strongly antigenic minor impurities. However, for quantitative assay purposes the reversed method would seem unsuitable from our experiment; both zones moved infinitely more slowly than could be expected on *Becker*'s theory from the diffusion coefficient of rabbit Ab. It is conceivable that the very high density of the precipitate in the upper precipitation zone may even have constituted a mechanical impediment to the diffusion of Ab. Roughly straight lines were obtained when plotting x against $\sqrt{t}$, but the relation of rate of diffusion of Ab to Ab-concentration in the upper layer would appear to be a complicated one that has not been covered by the available theoretical or empirical equations.

Oudin originally applied the term "Simple diffusion technique" to his method on the assumption that any diffusion of the Ab may be neglected in comparison with that of the Ag for sufficient Ag excess Although, as he states himself, this is clearly not the case, especially not for low Ag to Ab ratios, it is convenient to preserve the term "double" diffusion for methods in which Ag and Ab diffuse towards each other through an initially empty layer of gel. I have adopted *Oudin*'s classifications throughout, but have translated "diffusion simple" by "single diffusion", which would seem to be in accordance with his ideas.

Experimentally, *Oudin*'s method is a little tedious since delicate measurements have to be taken over prolonged periods; from 3 to 14 days are necessary for assay purposes, depending on the system. During such periods there is a danger that the reactants may deteriorate or reservoirs get exhausted, both of which may introduce errors.

Nevertheless, the method has already proved most valuable in a number of immunological systems (*Telfer*, 1953, *Oudin*, 1952, *Wodehouse*, 1954) and will continue to be so for the determination of physical constants of soluble antigens that are difficult to isolate and also to throw light on Ag-Ab reactions in general (*Munoz* et al., 1951, *Rubinstein*, 1954, *Spiers* and *Augustin*, 1957, *Augustin*, *Hayward* and *Spiers*, 1957).

2. *Simple Double Diffusion*

Double diffusion methods where both reactants diffuse into a neutral gel layer, would appear to offer certain advantages for purposes of immunological assay. Based on a suggestion of *Oudin* (1946), *Oakley* and *Fulthorpe* (1953) were the first to develop a quantitative double diffusion method in tubes which they used for measuring *antibody*.

There are three layers (fig. 2). The bottom layer contains Ab in gel as in *Oudin*'s single diffusion method; this is overlayered with an empty gel layer containing neither reactant, and a solution of Ag is put on the top of this. The two reactants diffuse towards each other to form a line where they meet in suitable concentrations. The line will move up or down according to whether Ag or Ab is in excess of the equivalence ratio (assuming equal diffusion coefficients). For any particular Ag-Ab system of given concentrations, the position of the line will be defined for any fixed time. If the given Ag solution forming the top layer is replaced in an otherwise identical set-up by an aliquot of the same Ag solution, but after it has been incubated with a known amount of standard antiserum, the diffusable Ag-concentration is reduced, and the line must appear higher up than before. *Oakley* and *Fulthorpe* measure Ab-concentrations by comparing them for their power to produce the same displacement of line as the known standard of the same system. There is, therefore, only one straightforward comparison of the positions of the lines in a series of tubes, usually after overnight incubation. The method is thus less cumbersome than *Oudin*'s single diffusion method, and it is irrelevant which reactant has the higher concentration. On the other hand, we are again dealing with moving lines, and the reactants must therefore once more be present in fairly high concentrations to give visible precipitation lines.

One ratio of the initial concentrations of the reactants exists which gives a stationary line, namely when Ag and Ab are present in equivalence concentrations (*Dean* and *Webb*, 1926). The density of this particular line will depend upon the total amounts of Ag and Ab present, and *not* on their concentrations. According to *Preer*'s approximate theoretical treatment (*Preer*, 1956) a fixed line is produced if $C_{01} = C_{02}$ where C_{01} and C_{02} represent the initial concentrations of the reactants in equivalent units. *Spiers* (1957),

taking into account the mutual anihilation of Ag and Ab in the plane of reaction (precipitation "line"), found that a stationary line formed when

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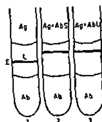


Fig. 2

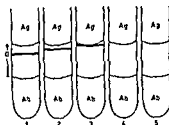


Fig 3

Fig 2 Simple double diffusion method by *Oakley* and *Fulthorpe*. An empty layer E is interposed between the Ag layer Ag and the Ab layer Ab (tube 1). A precipitation line L forms in the middle layer after a suitable time of incubation. In tube 2 the concentration of free Ag in the top layer has been reduced by allowing it to react with a standard quantity of Ab, the line appears higher up the tube Tube 3 shows the line in the same position as tube 2 after reaction of the Ag with a dilution of an Ab solution of unknown concentration, the dilution used in the preparation of the 3rd tube contains as much Ab as the standard. Volume changes due to the Ab additions are assumed to have been suitably adjusted and taken into account

Fig 3 Gradient diffusion method by *Augustin* and *Hayward* As in the method by *Oakley* and *Fulthorpe* neutral gel is layered above the Ab layer. After over-night incubation (for establishing a suitable gradient across the middle layer) decreasing concentrations of Ag have been added to a series of such equally treated tubes. Lines form higher and higher up the tube until a line touches the meniscus (3rd tube, indicator tube) The Ag concentrations in tubes 4 and 5 are too low to give a line in the middle layer. The concentrations of unknown Ag solutions are adjusted until a tube is obtained which also shows a line in the indicator position

There is a further point. The rate of movement of the precipitation line in the middle must vary according to the ratio of the initial concentrations and will be zero in one particular instance as already pointed out. Therefore, a given fixed addition of Ab to the top layer may produce variable displacements in the position of the original Ag-Ab precipitation line, i.e. the sensitivity of the method must vary with the Ag-Ab ratio of the standard tube. *Oakley* and *Fulthorpe* have left ratios as well as concentrations entirely undefined. Also the further away from the equivalence ratio the

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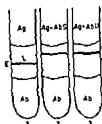


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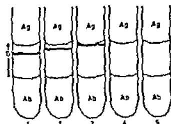


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There is a further point. The rate of movement of the precipitation line in the middle must vary according to the ratio of the initial concentrations and will be zero in one particular instance as already pointed out. Therefore, a given fixed addition of Ab to the top layer may produce variable displacements in the position of the original Ag-Ab precipitation line, i.e. the sensitivity of the method must vary with the Ag-Ab ratio of the standard tube. *Oakley* and *Fulthorpe* have left ratios as well as concentrations entirely undefined. Also the further away from the equivalence ratio the

2. *Simple Double Diffusion*

Double diffusion methods where both reactants diffuse into a neutral gel layer, would appear to offer certain advantages for purposes of immunological assay. Based on a suggestion of *Oudin* (1946), *Oakley* and *Fulthorpe* (1953) were the first to develop a quantitative double diffusion method in tubes which they used for measuring *antibody*.

There are three layers (fig. 2). The bottom layer contains Ab in gel as in *Oudin*'s single diffusion method; this is overlayered with an empty gel layer containing neither reactant, and a solution of Ag is put on the top of this. The two reactants diffuse towards each other to form a line where they meet in suitable concentrations. The line will move up or down according to whether Ag or Ab is in excess of the equivalence ratio (assuming equal diffusion coefficients). For any particular Ag-Ab system of given concentrations, the position of the line will be defined for any fixed time. If the given Ag solution forming the top layer is replaced in an otherwise identical set-up by an aliquot of the same Ag solution, but after it has been incubated with a known amount of standard antiserum, *the diffusable Ag-concentration is reduced*, and the line must appear higher up than before. *Oakley* and *Fulthorpe* measure Ab-concentrations by comparing them for their power to produce the same displacement of line as the known standard of the same system. There is, therefore, only one straightforward comparison of the positions of the lines in a series of tubes, usually after overnight incubation. The method is thus less cumbersome than *Oudin*'s single diffusion method, and it is irrelevant which reactant has the higher concentration. On the other hand, we are again dealing with moving lines, and the reactants must therefore once more be present in fairly high concentrations to give visible precipitation lines.

One ratio of the initial concentrations of the reactants exists which gives a stationary line, namely when Ag and Ab are present in equivalence concentrations (*Dean* and *Webb*, 1926). The density of this particular line will depend upon the total amounts of Ag and Ab present, and *not* on their concentrations. According to *Preer*'s approximate theoretical treatment (*Preer*, 1956) a fixed line is produced if $C_{01} = C_{02}$ where C_{01} and C_{02} represent the initial concentrations of the reactants in equivalent units. *Spiers* (1957),

layer prior to adding the Ag-solution in a concentration such that the precipitation line forms as a tangent to the upper meniscus of the (originally) neutral gel layer (fig. 3). Such a tangent to the meniscus provides an easily recognized reference position. The establishment of the Ab-gradient across the middle layer makes it possible for Ag and Ab to be in very low concentrations when they first meet at the interface. Furthermore this gradient has been adjusted so that the Ag-Ab ratio at the meniscus is very low. The lower the Ag-Ab ratio and the absolute Ag concentration the greater will be the displacement caused by an alteration in the Ag concentration in the top layer (fig. 4), i.e. the greater will be the sensitivity of the method. Equally sensitive conditions could have been achieved by *Oakley* et al. by suitably adjusting the concentrations of top and bottom layer but for practical limitations; at low absolute concentrations, it would take an inordinately long time for the reactants to meet in the middle layer and precipitation might never become visible. The prior establishment of the Ab-gradient in our method overcomes these difficulties. Not only do the reactants meet immediately after addition of the Ag but the line – once formed at or near the meniscus – remains fixed in this position indefinitely, although its density increases with time. The final density that can be reached depends upon the total amounts of the reactants, and not on their concentrations. The latter property is shared with the stationary line of simple double diffusion for equivalent concentrations of the reactants. Indeed the Ab-gradient is arranged such that equivalence conditions prevail until most of the Ag in the top layer has been used up. For practical purposes, the line in the meniscus provides then a "sink" (in the hydrodynamical sense) into which Ag and Ab disappear by reacting with each other. In this way the concentration of Ag-Ab complexes increases with time until all the reactant in the top layer is used up. This will occur very quickly. Because of the large Ab-reservoir in the bottom layer the density of the line will increase still further over a longer period of time; for under the influence of an ever increasing tide of Ab across the precipitation line (in the rabbit system, Ag-Ab complexes insoluble in excess Ab, fig. 1) Ag-Ab complexes of the highest possible Ab/Ag ratio will form, as discussed for a "reversed" *Oudin*-test, with Ab in the top layer.

initial Ag and Ab concentrations are the more spread will occur in the precipitation line either at the top or the bottom edge of the line according to which of the reactants is in excess. By setting up a series of tubes with varying Ag-Ab ratios including the equivalence point it is possible to find the equivalence tube which will exhibit the sharpest and most narrow line. This has been used by *Boerma-Westendorp* (1956) as another method for estimating Ag-concentrations while keeping the other reactant constant. However, the reactants must be present in rather high concentrations to exhibit this phenomenon clearly.

3. *Double Diffusion with Pre-Incubation. Gradient Diffusion Method*

We have attempted to improve upon the method of *Oakley* and *Fulthorpe* by establishing an Ab-gradient across the middle

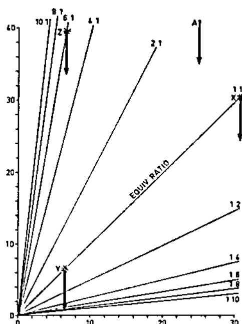


Fig 4. Sensitivity of double diffusion methods in relation to the initial Ag-Ab concen-

1. As near as possible to the initial Ag concentration in the top layer; a reduction

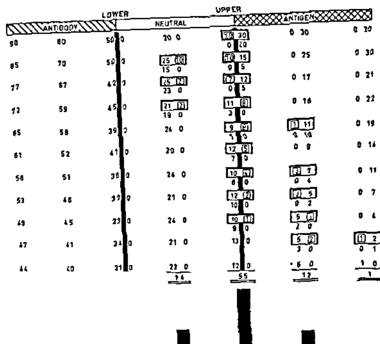


Fig 6. Formation of the precipitation line in the gradient diffusion method Very much simplified Schematic representation. Figures in heavy type represent Ab concentrations, figures in lighter type Ag concentrations Reaction is assumed to take place where equal amounts of Ag and Ab meet. The reacted amounts are edged around inside the heavily out lined boxes and are assumed to be lost to further diffusion processes The bulk of Ag-Ab precipitation is seen to have taken place in the meniscus For further details see text.

We are still in the process of developing a mathematical theory that will account for all our data. However, a crude diagramatic representation of the diffusion and precipitation processes involved in our method is shown in fig 6. The reaction tube is shown lying on its side and is imagined to be divided into a number of cells. The Ab gradient through these cells, after pre-incubation of bottom and middle layer, is assumed to be 90, 80... to 50 units at the lower meniscus, and 20 in the middle layer to 10 at the upper meniscus when the Ag is added. The Ag has to be added at a concentration higher than the equivalent Ab concentration in the meniscus. If equivalent concentrations of Ag and Ab are allowed to meet at a curved meniscus they divide the height of the meniscus between them to form a straight line cutting the meniscus. This has been established by us experimentally by direct

In practice the Ab-gradient is established very simply by incubating the Ab layer with the "empty" middle layer overnight prior to the addition of the Ag. The length of the middle layer is critical; too high an Ab-gradient near the upper meniscus will prevent the formation of a stationary line.

Ag units in top layer	Middle layer Length in cm	Appearance of Ag Ab ppt in middle layer Time in hours after addition of Ag			Hence units of Ag in indicator tube
		1	33	57	
12000 6000	0 3				6000
6000 3000	0 4				about 4000
3000 1500	0 5				about 2000
6000 3000 2000 1500	0 6				1500

Fig. 5 Gradient double diffusion method Position of Ag-Ab precipitation line in relation to length of middle layer The Ag concentrations in the top layer have been suitably adjusted so as to continue to obtain lines in the indicator position.

Fig. 5 shows that the position of the line depends indeed rather delicately on the rate of Ab-increase across the middle layer. In a series of experiments middle layers of variable length were used while keeping everything else constant. If the middle layer is too short, i. e. the rate of increase of Ab concentration is too rapid, not a line but a diffuse layer of precipitation with a moving leading edge occurs.

at the edges of the meniscus will be bigger than at the point where the horizontal tangent touches the meniscus. Therefore, under conditions of Ag excess (even if temporary) the line may form tangentially to the meniscus and Ag-Ab complexes in the edges of the meniscus may either never form in the first place or may re-dissovle. Curved lines may however form with fast moving reactants in too high concentrations in the bottom layer. We did obtain such curved lines in our first attempts of employing Ag in place of Ab in the bottom layer in too high a concentration (*Hayward* and *Augustin*, 1957).

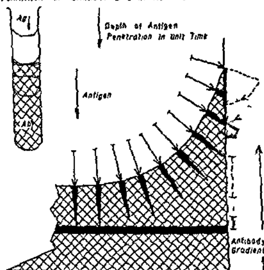


Fig 7. Diagrammatic representation of the formation of the straight Ag-Ab precipitation lines in gel diffusion tubes with curved menisci.

By varying the size of the top layer (from 0.1 to 4 cm) and keeping the concentrations of the reactants constant we have confirmed experimentally that the position of the line depends on concentration only while the density of the line increased with increase in total amounts of reactants. This has enabled us to measure smaller quantities of Ag than is possible by the *Oudin* method, or indeed by any method with constantly moving lines. Moreover, as the position of the line is fixed for all times within the first hour or so of adding the Ag, subsequent deterioration

and indirect means (*Augustin, Hayward* and *Spiers*, 1957). This over-simplified diagram assumes an Ag concentration of 30 units. All the concentrations are expressed in equivalent units and only precipitation at equivalence is considered. Therefore the 30 units of Ag will cancel 10 units of Ab, 20 units of Ag and zero units of Ab remaining. The subsequent diffusion process is then represented diagramatically by allowing half the units of each cell to flow in a period of time t into the cells on either side. After completing the next line by this procedure Ab and Ag units are cancelled out where they meet in equal concentration in the same cell. The diffusion process is then continued a further state, etc. Consecutive horizontal lines on the diagram thus give states of diffusion processes in consecutive units of time. The reacted Ag-Ab complexes are ringed round inside the big square and are summed up at the bottom of each cell. Precipitation appears to take place in three main stages, commencing with a swing forward into the middle layer, the main precipitations taking place in the meniscus, with a subsequent swing back into the Ag layer. In this diagram over 60% of the total available Ag is precipitated in the meniscus.

Although this is only a very simplified representation, the main features are borne out by our experiments. When the formation of precipitation lines is followed from the beginning one finds with narrow middle layers (fig. 5) that the edges of the meniscus are completely filled in with precipitate, but that these edges eventually are completely cleared of precipitate and that the final line is in the meniscus only. This again pre-supposes a relative Ag-excess at some stage. On increasing the length of the middle layer the intial precipitation in the edges of the meniscus becomes less and less apparent, simply because the Ab concentration there is very low, but the final outcome is still the same – a stationary sharply defined line in the meniscus.

Fig. 7 is a qualitative attempt to explain the formation of straight precipitation lines rather than curved lines following the shape of the meniscus. Part of the upper meniscus is enlarged on the right. As a first approximation diffusion of Ab is neglected and the diffusion of Ag is regarded as free diffusion at right angles to tangents of the meniscus. In a given time the thrust of Ag across the meniscus must at some point become bigger than the distance of the meniscus from the tube wall. There will thus be a residual downward component of the Ag thrust, i.e. the Ag flow

(*Elek*, 1949). The existence, in *Feinberg*'s plates, of Ab even behind the Ag cups favours this still more According to *Feinberg* as much as 72 hours are needed to establish a suitable Ab gradient before the Ag may be added.

Summary

1 The "gradient" double diffusion method of *Augustin* and *Hayward* is compared critically with the "simple" double diffusion method of *Oakley* and *Fulthorpe* and *Oudin*'s single diffusion method. The reasons for the greater sensitivity of the gradient diffusion method are pointed out.

2 Theories describing Ag-Ab behaviour in single and double diffusion methods are discussed and attempts are made to elucidate the mechanism of reaction in the gradient diffusion method.

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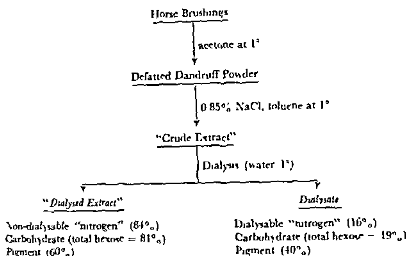
of the Ag solution cannot affect the position of the line and will only result in a reduction of line density. This method is therefore particularly suited for the assay of unstable solutions.

The method has been used successfully for the estimation of Ab also, either indirectly, similarly to *Oakley* et al., but also directly. Difficulties as with the attempts of measuring Ab by means of the single diffusion assay were not encountered (*Hayward* and *Augustin*, 1957).

Fractions of a μg of Ovalbumin could be assayed by means of this gradient diffusion method and 0.3 Lf of diphtheria toxoid while 5 to 7 antitoxic units as the internal reactant bring one to the limits of visibility in the *Oudin*-test; i.e. while 7 Lf of diphtheria toxoid will give a visible line, concentrations of $>$ 10 Lf units will be required for assay purposes.

Finally, a few words may be added with respect to single and double diffusion assays using plates instead of tubes. A great number of such plate methods have been described (*Elek*, 1949, *Gell*, 1956, *Augustin*, 1956, *Augustin* and *Hayward*, 1956, *Hayward* and *Augustin* 1957, *Feinberg*, 1956) but most of them are of a semi-quantitative rather than a quantitative nature. This is not surprising since it is much more difficult to ensure complete evenness of a comparatively large area of gel than to adjust the height of a column of gel in a tube. Also, Petri dishes have notoriously uneven bottom surfaces. The most reliable of these methods is probably that by *Feinberg*, who has gone to great trouble to standardise this procedure by carefully choosing his Petri dishes and employing a special cutter manufactured with great accuracy for cutting out the Ag and Ab reservoirs. The principle of the test has been taken from our gradient double diffusion method (*Augustin* and *Hayward*, 1955), i.e. an Ab gradient is established from a central Ab cup before filling the exactly equidistant peripheral cups with Ag solutions; once more stationary lines are formed. However the limited reservoirs of the reactants make it impossible to use this arrangement to increase the sensitivity of the test. The deliberately unbalanced layout of the test should not be used for qualitative analyses as it encourages the formation of artefacts such as the crossing of precipitation lines although they belong to the same Ag-Ab system. This may take place where cups of high and low Ag concentrations are adjacent when one precipitation line forms before the other and the reservoirs get exhausted

Fig 1 100 g portions of defatted powder were extracted with saline (1 litre), toluene (100 ml) being added as preservative.



other allergen systems. Until pure allergen preparations are available it will not be possible to form an adequate picture of the biochemical reactions involved in inhalant-type hypersensitivity, nor to correlate allergenic activity with any peculiarity in chemical constitution (if, indeed, such a relationship exists).

The advent of physico-chemical methods such as electro-

protein mixtures Moreover, such mild procedures are far less deleterious than the earlier methods employing strong acid, alkali and organic solvent precipitants Several of these more modern techniques have been applied to the fractionation of horse dandruff extract with the aim of accounting for each individual constituent and considering its possible rôle as allergen In this way it was hoped to establish whether there was only one substance or several associated with biological activity.

At various stages in the work to be described crystalline horse serum albumin (HSA) has been used as a reference substance. The reason for this will become apparent later, when the supposed

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The Use of the Gel-Precipitation Technique in the Identification of Horse Dandruff Allergen, and in the Study of the Serological Relationship between Horse Dandruff and Horse Serum Proteins

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Introduction

Application of the gel-precipitation technique to the study of the horse dandruff protein system can only be illustrated fully against the background of the preceding chemical studies described in detail in a recent paper in the Biochemical Journal (1). In this work the *Ouchterlony* technique has been mainly employed as a complementary qualitative method for comparing the antigenic compositions of dandruff protein preparations and identifying individual constituents. It has proved particularly suitable for this purpose, at a stage when physico-chemical techniques failed through lack of sensitivity.

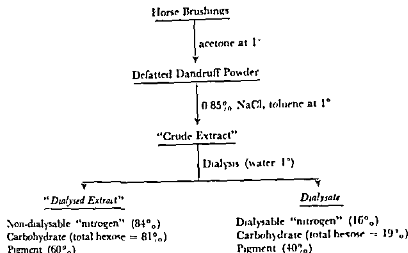
Work on the horse dandruff system has been conducted with two main aims in view:

1. Preparation of a pure allergen for chemical characterisation and therapeutic purposes;

2. Establishment of the immunochemical mechanism involved in the severe reaction sometimes experienced by horse-sensitive asthmatics on receiving their first injection of therapeutic horse serum.

In this way it was hoped to provide a less empirical basis for the clinical findings on horse-asthma and, possibly, improve the accuracy of diagnosis of this type of hypersensitivity. Moreover, it seemed likely that the results of experiments on the particular model chosen might assist in a better understanding of

Fig 1 100 g portions of defatted powder were extracted with saline (1 litre), toluene (100 ml) being added as preservative



other allergen systems. Until pure allergen preparations are available it will not be possible to form an adequate picture of the biochemical reactions involved in inhalant-type hypersensitivity, nor to correlate allergenic activity with any peculiarity in chemical constitution (if, indeed, such a relationship exists).

The advent of physico-chemical methods such as electrophoresis and ultracentrifugation in the last twenty years (a period, incidentally, during which little fresh has been learnt about animal dandruff allergens) has led to the unravelling of many complex protein mixtures. Moreover, such mild procedures are far less deleterious than the earlier methods employing strong acid, alkali and organic solvent precipitants Several of these more modern techniques have been applied to the fractionation of horse dandruff extract with the aim of accounting for each individual constituent and considering its possible rôle as allergen In this way it was hoped to establish whether there was only one substance or several associated with biological activity

At various stages in the work to be described crystalline horse serum albumin (HSA) has been used as a reference substance. The reason for this will become apparent later, when the supposed

serological cross-reactivity between horse dandruff and horse serum proteins is considered.

Preparation of Dialysed Extract

The extracts used throughout were obtained by the procedure outlined in Fig. 1.

Crude horse brushings were freed from hair by suspension in acetone at 1° C and then pouring through a Buchner funnel. After standing in cold acetone for about 1 hour, the resultant defatted powder was separated, dried and extracted with 0.85% saline containing toluene as preservative. Removal of insoluble epidermal material after three days left "crude dandruff extract".

The light brown opalescent protein solution obtained after prolonged dialysis was termed "dialysed dandruff extract". It usually contained about 1 g protein/100 ml, as well as pigment and carbohydrate. About 40% of the pigment in crude extract together with low molecular weight substances (peptides, amino acids, sugar and salts) was removed by the dialysis process. On the other hand, 90% of the carbohydrate (estimated as hexose) was found to be conjugated to protein as mucoprotein. The rest was in the form of mucopolysaccharide No nucleoprotein or lipoprotein was detected.

The protein, however, was the important constituent. *Squire* (2) had shown that all skin-reactivity could be removed from dialysed extract by common protein precipitants such as trichloroacetic acid, and also, by precipitation at pH 5.0 with 22.5% (W/V) Na_2SO_4.

The first step, therefore, was the assessment of the homogeneity of this active protein material

Electrophoresis and Ultracentrifugal Analysis

Electrophoresis

Results obtained by free-solution electrophoresis of dialysed extract in barbitone buffer (pH 8.6, I = 0.1) are shown in Fig. 2A.

Three components separated and these have been lettered arbitrarily (as a, b and c) in decreasing order of mobility. Relative concentrations were calculated only from the ascending pattern, as separation of the slowest *c* component was incomplete in the

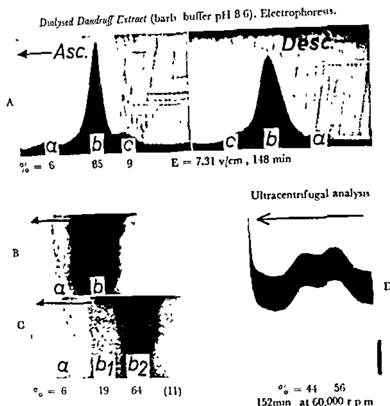


Fig 2 Free-solution electrophoresis was carried out in buffer of ionic strength 0 1, whilst buffer of half this strength was used in the paper-strip and ultracentrifugal analyses (figs 2A, B, C taken from Biochem J 1957, *65*, 582)

descending limb. It will be seen that these values (a = 5 6%, b = 85,3%, c = 9.1%) agree well with the corresponding values obtained by paper-strip analysis (Fig 2B) on 3 MM paper (in which no definite separation of *c* component was observed) This suggested that the proteins did not differ greatly in their contents of non-protein prosthetic groups. The splitting of the major b component into b_1 and b_2 (Fig 2C) which sometimes occurred in the solid medium, was not surprising in view of the asymmetry of the corresponding schlieren peak

Mobility

The b component was found to have a faster mobility (-8.03×10^{-5} as compared with $-6\,49 \times 10^{-5}$ cm^2 v^{-1} sec^{-1}) than five times

crystallised HSA in barbitone buffer (pH 8.6, I = 0.1). This could also be demonstrated by paper-electrophoresis of a mixture containing the two proteins, where the albumin was seen to migrate behind the dander constituent.

The iso-electric point of the b component was estimated from free-solution electrophoresis at three different pH values to be around pH 4.0.

Ultracentrifugal Analysis

In contrast with electrophoresis, ultracentrifugal analysis (in barbitone buffer; pH 8.6, I = 0.05 + 0.2 M. NaCl) only separated two components even after 152 mins. sedimentation at 60 000 rpm. (Fig. 2D). This finding was in agreement with preliminary results published recently by *Silver* and *Bookman* (3). The lighter fraction (56% of total) appeared to include the b_2 electrophoretic component. From the uncorrected sedimentation constant (2.5 S) of a purified b_2 preparation, which sedimented as a single boundary in barbitone buffer containing 0.2 M. NaCl, the approximate molecular weight of the major protein constituent was calculated to be of the order of the 34 000 value obtained from osmotic pressure measurements.

Salt Fractionation

Having revealed several protein constituents in dialysed horse dandruff extract, the next step was their isolation prior to assaying their skin reactivities. Salt fractionation was chosen in preference to the more elaborate low temperature-ethanol techniques evolved at Harvard.

Solubility Curve of Dialysed Horse Dandruff Extract in Ammonium Sulphate

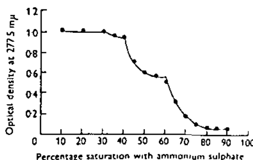


Fig. 3. (taken from Biochem J *65*, 582, 1957)

The solubility curve obtained from a small-scale quantitative fractionation of dialysed dandruff extract *with ammonium sulphate* (adjusted to pH 7.0 with concentrated *NH_3 solution) is shown in* figure 3. *When the* ultraviolet (277.5 m μ) absorptions (proportional to protein concentrations) of the supernatants were plotted against each salt concentration used for precipitation, the fractionation of three protein constituents was revealed (between 30–40%, 40–59% and 59–80% saturation with salt). This information proved a useful guide *in fixing the salt concentration limits in a* large-scale fractionation (outlined in figure 4).

It was interesting to find that no salt concentration limit *corresponded to 50% saturation with ammonium sulphate*, the accepted "dividing line" between serum globulins and albumin.

Ammonium Sulphate Fractionation of Horse Dandruff Extract

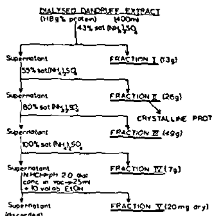


Fig 4 (taken from Biochem J *65*, 582, 1957)

Large-Scale Fractionation

In the fractionation scheme shown in figure 4, the figures in brackets refer to the wet weights of the *protein* ppts. (reckoned to be about 10 times dry wts) These values indicated a distribution of dandruff protein amongst the various fractions in agreement with that predicted from the solubility curve, the major portion being precipitated between 55–80% saturation with $(NH_4)_2SO_4$

crystallised HSA in barbitone buffer (pH 8.6, I = 0.1). This could also be demonstrated by paper-electrophoresis of a mixture containing the two proteins, where the albumin was seen to migrate behind the dander constituent.

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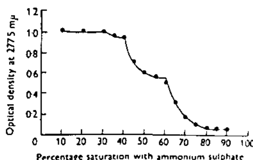


Fig 3 (taken from Biochem J *65*, 582, 1957)

Analysis of Salt-Fractions

Considering the other fractions, the first requirement was the assessment of their compositions in order to establish whether any fractionation had, in fact, been achieved. Free-solution electrophoresis was first used for this purpose, but was later abandoned in favour of the less time consuming paper-strip technique requiring much less material and providing an easier means of identifying the separated components It is possible, for instance, to split the strips lengthwise and stain one half for carbohydrate with periodic acid – *Schiff*'s reagent, thus locating glycoprotein constituents.

Paper-Electrophoresis

The results of analyses on whole dandruff extract and each salt-precipitated fraction are shown in figure 5. Hexose/nitrogen ratios (determined by the orcinol colorimetric and micro-Kjeldahl techniques) are included for comparison.

Although the splitting of the b component in the whole extract is not demonstrated on this occasion, it is clear that carbohydrate is only associated with the slower b_2 constituent. Considering the electrophoretic constituents in order of increasing solubility, the carbohydrate-free b_1 component was precipitated in salt-fractions I and II. Next, the carbohydrate-rich b_2 component was beginning to separate in fraction II, became predominant in fraction III and was still present in considerable amount in fraction IV. Finally, the fast *a* component (containing a little carbohydrate) began to separate in fraction III but was precipitated mainly in the most soluble fraction IV, in which a small amount of a slower component (d) was detected.

In view of its extremely high hexose/nitrogen ratio (8 62) and predominant carbohydrate properties, fraction V was termed a «mucopolysaccharide». In contrast, the crystalline dandruff protein was carbohydrate-free, although it possessed a similar paper-electrophoretic mobility to the carbohydrate-rich b_2 component

Identification of the protein constituents in each fraction was aided by comparative electrophoresis of 1% (W/V) solutions on a wide-sheet of *Whatmann* 3 MM paper (in barbitone buffer; pH 8 6, I = 0,05 at 10 ma for 8 hours). The results of such an analysis are illustrated diagrammatically in figure 6. They emphasise the

After repeated attempts, a crystalline protein was isolated from fraction II. The crystals were very fragile but appeared to be of hexagonal form (thus differing from the trigonal form of HSA crystals). Unfortunately, however, solutions of these crystals proved unreactive in skin tests on horse-sensitive subjects, so that hopes of having prepared crystalline allergen were not fulfilled.

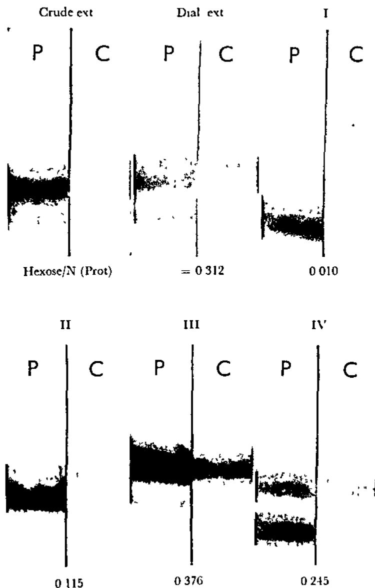
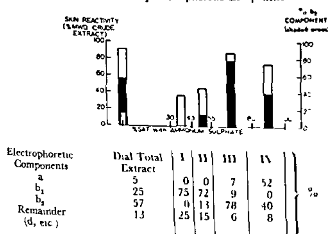


Fig 5. Comparison of paper-electrophoretic glycoprotein constituents of whole dandruff extract and salt-precipitated fractions (Barbitone buffer, pH = 8 6, I = 0 05, 2 ma/strip for 16 hours) (Taken from Biochem J, 1957, *65*, 582)

showed that there was a coefficient of variation of about 16% on a single measurement (when site and time of test variations were controlled). He was able to show, also, that an approximate linear relationship holds between mean wheal diameter and the logarithm of the histamine concentration over a 1-6 mm wheal size range It was apparent from the log. (histamine concentration) – mean wheal diameter curve that only a 2 fold increase in skin-reactivity is produced by about a 10 fold increase in dose of reactant This means that a difference in skin reactivity between two allergen preparations would have to be fairly great if it were to be revealed convincingly by the prick test. Whilst appreciating the short-comings of this test as a quantitative measure of allergenic activity, it is felt that it is as reliable as the alternative less direct methods, providing the standard error of the results obtained with each test solution is established.

Comparison of Skin Reactivities of Dandruff Extract and Salt Fractions with their Content of b_2 Electrophoretic Component



Electrophoretic Components	Dial Total Extract	I	II	III	IV	
a	5	0	0	7	52	%
b_1	25	75	72	9	0	%
b_2	57	0	13	78	40	%
Remainder (d, etc)	13	25	15	6	8	%

Fig 7 Skin reactiv... as percentages of
Relative paper-e
... of bound dye

Results

The results obtained by prick testing 5 horse-sensitive children (aged 9-13) with 0.1% (W/V) solutions of whole dandruff protein and its salt-fractions are shown in the form of a histogram in

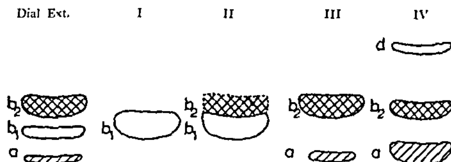


Fig 6 Comparative paper-electrophoresis of 1% (W/V) solutions of dialysed dandruff extract and salt-fractions in barbitone buffer (pH = 8.6, I = 0.05, current = 10 ma for 8 hours) The hatching indicates low carbohydrate content and the cross-hatching indicates high carbohydrate content (Taken from Biochem J 65, 582, 1957)

diversity of composition of the various salt-fractions, at the same time indicating that substantial fractionation had been achieved. It seemed likely, therefore, that comparison of the skin-reactivities of these fractions would provide information about the identify of the dandruff allergen and possibly lead to correlation of biological activity with a single component.

(Suppose, for example, that the sole allergen in dandruff extract was the b_1 component, then one would expect skin-reactivity to be confined to salt-fractions I and II).

Assay of Skin-Reactivities

Technique

Before describing the skin-test results, a brief description of the technique employed for determining allergenic activity should be given. This involved pricking drops of seitz-filtered protein solutions in saline, of comparable concentrations (usually 0.1 g/100 ml.) on a nitrogen or dry weight basis, into selected sites on a horse-sensitive subject's forearm. Tests were performed in duplicate and after 10 mins. the outlines of the resultant wheals were traced in ink on to stiff celluloid Finally, the areas of the enlarged tracings were determined by the method of "counting squares" (and more recently by weighing) and used for the calculation of accurate mean wheal diameters ("MWD" in results to be shown).

The reliability of this method has been demonstrated by *Squire* (2), who carried out repeated tests with histamine and

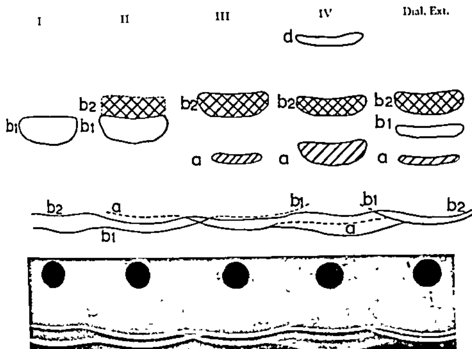


Fig 8 Comparison of paper-electrophoresis patterns shown in fig 6 with the corresponding gel-precipitation spectra obtained by using antiserum raised in rabbits to dialysed dandruff extract 1% (W/V) antigen solutions were tested

Gel-Precipitation Analysis

In fig 8 the antigenic spectra of 1% solutions of whole dandruff extract and its salt fractions have been aligned with their paper-electrophoresis patterns shown earlier in figure 6. (The antisera used in such analyses were raised in rabbits with a *Freund* adjuvant to a 5 0% solution of total non-dialysable dandruff protein). Side by side arrangement of antigen cups was preferred to the more common radial arrangement, in which identification of the lines was found to be more difficult

The main precipitin lines shown were identified by comparison with realtively pure proteins obtained by preparative electrophoresis (As many as seven lines have been observed on analysis of crude extract under certain conditions). It will be seen that the dialysed extract spectrum is comprised of three main lines, corresponding to the a, b_1 and b_2 electrophoretic components. Where superimposition of lines occurred, identification was supplemented

figure 7. Mean wheal diameters (MWD) have been expressed as percentages of the mean wheal diameters elicited by crude dandruff extract. An analysis of variance had previously shown that this way of expressing results was valid; besides indicating that the 5 individuals tested did not show variation in specific hypersensitivity to any particular dandruff protein constituent (1). In other words, the subjects all reacted in the same manner as far as specificity was concerned.

When one examines the mean reactivities of the various fractions it is found that, in addition to whole extract, fractions III and IV are the most potent skin-reactants, the former showing the highest potency of all the fractions tested. At the other extreme, fraction I showed relatively low reactivity (the mucopolysaccharide fraction V proved to be unreactive).

On comparing the electrophoretic compositions of the dialysed extract and salt-fractions, set out below the appropriate blocks in figure 7, one finds that the carbohydrate-rich b_2 component is predominant in whole extract (57% of non-dialysable protein) and in fraction III, as well as comprising a substantial (40%) proportion of fraction IV. On the other hand, none of this component could be detected by paper-electrophoresis of fraction I and relatively little (13%) in fraction II. The percentage distribution of the b_2 component amongst the various fractions is shown by the shaded areas in the blocks. It will be seen that skin reactivity parallels b_2 content closely.

These findings suggested therefore that the b_2 component was, in fact, responsible for allergenic activity, especially as it was impossible to associate skin reactivity with any other electrophoretic constituent. For instance, as mentioned earlier, if component b_1 were the allergen, fraction I would show activity in marked excess of fractions III and IV By similar reasoning, components *a* and *d*, predominant only in fraction IV, can be excluded as possible allergens.

If, however, one is to accept a "unitarian hypothesis" pertaining to allergenic activity of horse dandruff constituents, one must explain why fraction I, which is deficient in the supposedly allergenic b_1 component, shows small but measurable skin-reactivity. At this stage, it is fortunate to find available a more sensitive method of assessing protein heterogeneity, namely the gel-diffusion precipitin tests.

Table I

Comparison of the content of b_2 component in dialysed dandruff extract and salt-fractions determined by quantitative paper-electrophoresis and gel-precipitation (Content of b_2 component in whole extract taken as 100 in gel-precipitation analyses).

Fraction	Hexose/N Ratio	% b_2 component (paper electrophoresis)	% b_2 component gel-diffusion)	Skin Reactivity %, M.W.D. Crude Ext.
Dial Extract	0 31	57	100	91
I	0 01	0	1	37
II	0 12	13	18	45
III	0.38	78	71	88
IV	0 25	40	10	77
V	8 62	0	0	—

Assay of Column Electrophoresis Eluates

Further evidence in support of the b_2 component being the dandruff allergen was obtained by skin-testing relatively pure antigen preparations obtained by column electrophoresis in starch and acetylated cellulose. In this way, for instance, a pure prepa-

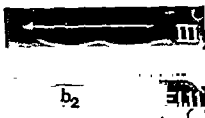


Fig 9 Immunoelectrophoretic comparison of starch electrophoresis eluate (El No 11) with parent salt-fraction III, using antiserum raised against crude dandruff extract. (Taken from Biochem J 65, 582, 1957)

ration of component *a*, which gave only one precipitin line on testing a 1% (W/V) solution with antiserum to crude dandruff extract, proved virtually unreactive Conversely, by careful selection of eluates from a starch electrophoresis run on the most potent salt-fraction (*III*), one could obtain relatively pure preparations of the b_2 component showing increased skin-reactivity as compared with the parent fraction. The purest preparation yet obtained by this process proved to be the most potent, possessing 150% of the skin-reactivity of fraction *III* (which itself was 88% as active as crude extract) Immunoelectrophoretic comparison of this eluate (*No. 11*) with its parent fraction III is illustrated in

by preliminary electrophoresis (in barbitone, pH 8.6, I = 0.05) in the direction of unaided diffusion. This treatment for example, drew out the *a* line from the b_1 line in the dialysed extract spectrum.

As in the comparative paper-electrophoresis picture, it is possible to trace each line through the antigenic spectra of the various fractions. Thus, one can observe the b_1 line, predominant in fractions I and II, fade out in fraction III. The *a* line, on the other hand, is first detected in fraction II, comes to the fore in fraction III and is in marked preponderance in fraction IV (where it splits). The detection of some of the b_2 component in fraction I, *serves to explain the skin reactivity of this fraction, which had* seemed anomalous on the basis of paper-electrophoresis alone.

As the b_2 line in the fraction I spectrum was much farther from the antiserum trough than it was in the other spectra and also less intense, it was concluded that the antigen was present in relatively small amounts in fraction I. (It will be recalled that the intensity and position of the precipitin line are dependant upon the antigen concentration). It was not possible to make any reasonable assessment of the relative concentrations of b_2 antigen in the other salt fractions from the position of this precipitin line, although the intensity of the line appeared to be greatest in the fraction III spectrum.

Quantitative tests

Preliminary measurements of the relative concentration of the b_2 antigen in dandruff extract and in its salt-fractions by means of the double diffusion precipitation technique, evolved by *Augustin* and *Hayward* (4), were in accordance with the relative b_2 component concentrations obtained from paper-electrophoresis (by assuming all constituents bind dye to the same extent). These values are compared in table 1 together with the skin reactivities of the fractions. Hexose/nitrogen ratios are also included. This table summarises, therefore, all the evidence pointing to the association of allergenic activity with the b_2 mucoprotein component of horse dandruff extract.

It has since been found possible to predict the order of skin reactivity of a series of protein fractions obtained by different procedures from their relative hexose contents *and the preponderance* of the b_2 line in their antigenic spectra.

Table I

Comparison of the content of b_2 component in dialysed dandruff extract and salt-fractions determined by quantitative paper-electrophoresis and gel-precipitation (Content of b_2 component in whole extract taken as 100 in gel-precipitation analyses)

Fraction	Hexose/N Ratio	% b_2 component (paper electrophoresis)	% b_2 component (gel-diffusion)	Skin Reactivity % M.W.D. Crude Ext
Dial Extract	0·31	37	100	91
I	0·01	0	1	37
II	0·12	13	18	45
III	0·38	78	71	88
IV	0·25	40	40	77
V	8·62	0	0	—

Assay of Column Electrophoresis Eluates

Further evidence in support of the b_2 component being the dandruff allergen was obtained by skin-testing relatively pure antigen preparations obtained by column electrophoresis in starch and acetylated cellulose. In this way, for instance, a pure prepa-

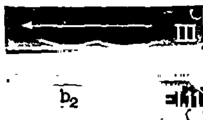


Fig. [illegible]

ration of component *a*, which gave only one precipitin line on testing a 1% (W/V) solution with antiserum to crude dandruff extract, proved virtually unreactive. Conversely, by careful selection of eluates from a starch electrophoresis run on the most potent salt-fraction (III), one could obtain relatively pure preparations of the b_2 component showing increased skin-reactivity as compared with the parent fraction The purest preparation yet obtained by this process proved to be the most potent, possessing 150% of the skin-reactivity of fraction III (which itself was 88% as active as crude extract) Immunoelectrophoretic comparison of this eluate (No. 11) with its parent fraction III is illustrated in

figure 9. It will be seen that one antigen line has been removed from the fraction III spectrum by the electrophoretic fractionation.

When, however, one studies the distribution of skin-reactivity (as indicated by tests on 3 horse-sensitive subjects) amongst various selected eluates corresponding to the main b_2 component peak of the elution pattern (fig. 10), one finds that activity is concentrated

Starch Columm Electrophoresis of Fraction III.

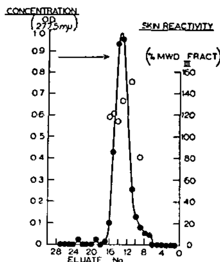


Fig 10. Skin-reactivities of selected eluates (o) are expressed as percentages of the mean wheal diameter elicited by the parent salt-fraction (precipitated between 55% and 80% sat of extract with $(NH_4)_2 SO_4$)

more on the leading edge of the curve. As is seen, on testing eluates of comparable protein concentration the most potent eluate is No. 11 and the activities of subsequent eluates fall off as the main curve is traversed. The most likely inference is that the b_2 peak is, in fact, composite, the faster of the two sub-fractions being associated with allergenic activity. This would account for an extra unidentified precipitin line which appeared in the antigenic spectra of eluates 12–16.

Further column electrophoretic analyses in acetylated cellulose tended to confirm the assumption that the b_2 component was heterogeneous. The type of pattern obtained is shown in figure 11. It became apparent that the main b_2 peak was composed of two incompletely separated components, temporarily designated as b_2 (F), the faster, and b_2 (S), the slower component Evidence

is now accumulating in support of the theory that only the faster b_2 (F) component is associated with allergenic activity.

Acetylated Cellulose Electrophoresis of Dandruff Protein Fraction III
(precipitated between 55–80% sat of whole extract with ammonium sulphate)

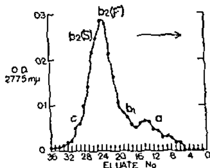


Fig 11 Barbitone buffer (pH 8 6, I = 0.05), 22 ma 21 hr.

When one re-examines the antigenic spectra of the crude salt-fractions, obtained by using weaker (0.5%) protein solutions, one can actually achieve splitting of the b_2 line, presumably into b_2 (F) and b_2 (S) As is seen from the precipitin patterns shown in figure 12, however, this does not affect the conclusions based on the results described earlier in which the b_2 (F) and b_2 (S) lines were not separated. The two lines remain close together, showing a fairly parallel distribution amongst the various fractions.

Preparation of Pure Allergen

The discovery of two antigens within the b_2 component has naturally complicated attempts to prepare the allergen in pure form. Nevertheless, it has served to emphasise the essential value of the gel-precipitation technique in assessing the purity of allergen preparations

An example of its use in this field is shown in figure 13 in which the degree of purification achieved by reprecipiting salt-fraction III three times and then sub-fractionating is compared with that obtained by column electrophoresis of the crude salt-fraction It will be seen that there is little difference between the spectra of the 60–70% and 70–80% saturated ammonium sulphate fractions and the crude fraction III spectrum. (In fact the main

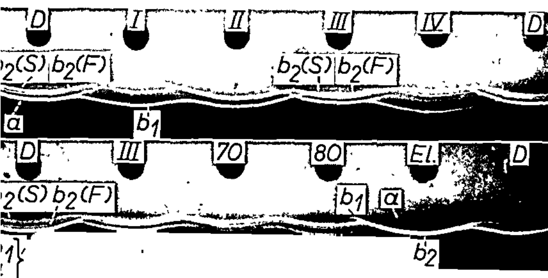


Fig 12 Comparison of the antigenic spectra of 0 5% (W/V) solutions of dandruff extract (D) and salt-fractions (I–IV), using antiserum raised against dialysed dandruff extract

Fig 13 Comparison of antigenic spectra of 0 5% (W/V) solutions of dandruff extract (D), salt-fractions III (55–80% saturation), 70 (60–70% saturation) and 80 (70–80% saturation) with a selected acetylated cellulose electrophoresis eluate, using antiserum *raised against dialysed dandruff extract*

purification achieved by reprecipitation appears to have been removal of pigment). On the other hand, the selected acetylated cellulose electrophoresis eluates were considerably more pure, being contaminated only with some b_1 component. In addition the electrophoresis process removed pigment, which travelled ahead of the b_2 peak.

This example illustrates the superiority of the gel-precipitation technique over currently used physico-chemical methods for assessing protein heterogeneity. It was not possible, for instance, to detect any heterogeneity of the acetylated cellulose electrophoresis eluate by subsequent paper-strip analyses over a wide pH range (4.6–10.6). Moreover, it was equally impossible to differentiate clearly between the b_2 (F) and b_2 (S) components by this technique

Serological Relationship between Horse Dandruff and Horse Serum Proteins

From results obtained by prick testing horse-sensitive subjects and carrying out optimal proportion titrations with rabbit anti-

sera to horse dandruff and horse serum proteins, *Squire* (2) had concluded that the severe reactions experienced by some horse-sensitive asthmatics when first injected with therapeutic horse serum could best be explained in terms of an immunological cross-reaction This, he suggested, probably took place because the antigenic dandruff protein was related to, but not identical with, horse serum albumin

The possibility of such a structural relationship existing between the horse dandruff and serum proteins was borne in mind throughout the studies on the horse dandruff system, just described. Every physico-chemical method employed, however, revealed an appreciable difference between the properties of the major dandruff protein and horse serum albumin. It will be recalled, for instance, that there was a marked difference in the electrophoretic mobilities of the two proteins. The magnitude of this difference implied that the relationship between horse dandruff and horse serum proteins was less close than, say, that reflected in differences in the electrophoretic mobility of one protein (for example ovalbumin) obtained from different species (5).

Having successfully applied the gel-precipitation technique to the identification of the allergen in horse dandruff extract, it seemed possible that its application here would throw new light on the nature of the relationship between horse dandruff and horse serum protein and establish with which dandruff antigen the serum albumin cross-reacted Such information could not be readily obtained by means of the *Dean* and *Webb* optimal proportion titration method (6), which relies on well separated precipitation optima if antigenic heterogeneity is to be detected

Results

When an *Ouchterlony* plate test (fig 14) was set up, comparing the reactions of 1% (W/V) dandruff protein solutions and 10% HSA solutions with rabbit antiserum raised to crude horse dandruff extract, it was clearly evident that the main albumin line [illegible]

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Even more definite results were obtained by studying the reactions between concentrated dandruff protein solutions and

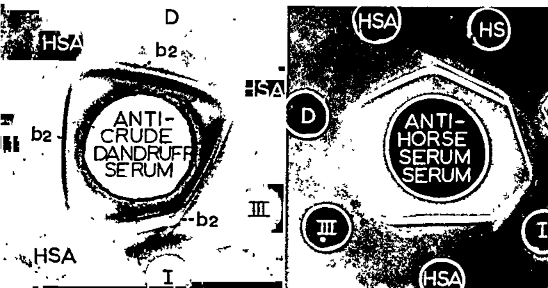


Fig 14 Comparison of the antigenic spectra of 1% (W/V) solutions of dialysed dandruff extract (D), salt-fractions I and III and starch-electrophoresis eluate No 11 with the spectrum of a 10% (W/V) solution of horse serum albumin using antiserum to crude dandruff extract

Fig 15. Comparison of the antigenic spectra of 1% (W/V) solutions of horse serum (HS), horse serum albumin (HSA) and horse serum globulin (HSG) with the spectra of 20% (W/V) solutions of dialysed dandruff extract (D) and salt-fractions I and III using antiserum raised to whole horse serum

antisera raised to horse serum and horse serum albumin A typical precipitin picture is shown in figure 15. The centre cup contained neat anti-horse serum and the outer cups contained 1% solutions of horse serum protein fractions and 20% solutions of dandruff protein fractions (total protein, D, and salt-fractions I and III). It will be seen that the serum albumin line runs from the horse serum spectrum through the spectra of the crystalline HSA and dandruff protein fraction III (precipitated between 55–80 saturation with ammonium sulphate) but ends when reaching the spectrum of the dandruff protein fraction I (precipitated between 30–43% saturation with ammonium sulphate). All this is in accordance with there being horse serum albumin antigen in dandruff extract. Moreover, the least soluble dandruff protein fraction I, although not containing any HSA, showed a serum globulin line in its spectrum.

Hence, one must conclude that dandruff extract contains small amounts of horse serum albumin and globulin. Further evidence of its contamination with serum albumin was obtained from experiments carried out with absorbed antisera.

Absorption Experiments

All antibody to HSA could be removed from homologous rabbit antiserum (diluted 1 : 1) by absorption with equal volumes of either.

1. 0.01% (W/V) HSA solution (i.e. 1 ml. antibody required 200 γ HSA for optimal precipitation) or

2. 20% (W/V) total dandruff protein solution,

so that no further precipitation occurred on testing the supernatants with dilute (1/100, 1/1000, 1/10 000) HSA solutions (or for that matter, with antiserum). The 20% total dandruff protein solution recovered from such experiments (now diluted with an equal volume of rabbit serum) gave the usual type of antigenic spectrum on testing a ten-fold diluted solution with anti-serum to dialysed dandruff extract. 2% total dandruff protein solution diluted with an equal volume of normal serum was set up as a control and gave precisely the same spectrum, indicating that no dandruff antigen had been removed by first using the dandruff extract to absorb antibody to HSA.

These results were interpreted as corroborative evidence in favour of the gel-precipitation findings. Hence it appears that the serological relationship between horse dandruff and horse serum proteins is due to the admixture of small amounts of serum albumin (and globulin) with the dandruff antigens and not the result of any cross-reactivity between the two types of protein.

Discussion

Application of gel-precipitation to the study of the horse dandruff system has proved useful in determining the nature of the allergen and also in "quality control" during successive stages of purification. It is evident that the technique is particularly valuable when combined with physico-chemical methods such as selective salt-precipitation and electrophoresis.

The two main advantages of the gel-precipitation technique illustrated by this work are:

a) Its sensitivity e.g. it has explained the skin-reactivity of a salt-fraction containing too low a content of allergen for detection by paper-electrophoresis;

b) Its resolving power e.g. in revealing a greater number of constituents in dandruff protein fractions than was evident from electrophoretic analysis; in demonstrating that even the b_2 component (the purest allergen yet isolated) was composite and in establishing the presence of traces of horse serum proteins in dandruff extract. The last of these findings offers a reasonable basis for the well-known tendency of horse-asthmatics to be sensitised, also, to horse serum and its products.

The standardisation of any allergen extract must ultimately depend upon a biological test in the hypersensitive patient. Nevertheless, in this work on horse dandruff extracts, which have always been handled with due regard for the instability of proteins, no evidence has been obtained (contrary to the findings of *Augustin* working with pollens [7]) to suggest that the appropriate gel-precipitation line is not a very useful indication of the presence of active allergen. The preliminary results of quantitative gel-precipitation assays on dandruff protein fractions, involving measurement of the b_2 precipitin line, confirmed this view.

Summary

Physico-chemical and immunological techniques have been applied to the study of horse dandruff extract in an attempt to identify its allergenic constituent. The gel-diffusion precipitation test proved useful in the analysis of protein fractions at a stage where other methods such as electrophoresis had been extended to their limits of sensitivity.

Three main protein constituents were revealed in dandruff extract by both free-solution and paper-strip electrophoresis in barbitone buffer (pH 8.6, I = 0.05–0.10), the latter technique effecting a splitting of the major component in some instances. Ultracentrifugal analysis, on the other hand, only separated two components from whole extract.

The antigenic compositions of fractions obtained by ammonium sulphate precipitation and column electrophoresis were compared by the *Ouchterlony* technique, employing rabbit antiserum raised

against whole horse dandruff extract. Skin reactivities were determined by the quantitative prick-testing of horse-sensitive patients.

Carbohydrate estimations and paper-electrophoresis showed that allergenic activity could be associated with a single mucoprotein constituent (b_2), comprising about 65% of the total dandruff protein and precipitating mainly between 55–80% saturation with ammonium sulphate. This finding was substantiated by carrying out skin tests with purer fractions.

Recent column electrophoresis (acetylated cellulose) and gel-precipitation analyses have revealed heterogeneity of the b_2 component. This again demonstrated the usefulness of the gel-precipitation technique in controlling the purity of allergen preparations.

Results obtained from gel-precipitation and specific absorption experiments indicated that the serological relationship between horse dandruff and horse serum proteins is due to the presence of small quantities of serum albumin and globulin in the dandruff material.

Acknowledgements

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The Psychiatric Approach to Allergic Disorders

By D LEIGH, London

(Manuscript not received)

Hayward, B. J. and *Augustin, R.* · Int. Arch. Allergy *11* 192—205 (1957)

Quantitative Gel Diffusion Methods for Assay of Antigens and Antibodies

By B. J. Hayward and R. Augustin*

From the Wright-Fleming Institute, St. Mary's Hospital Medical School, London.

In 1955 *Augustin* and *Hayward* described a gradient double diffusion method in tubes for the assay of antigens (Ag). The theoretical aspects of this test together with a critical comparison with other gel diffusion techniques have been discussed in a previous paper (*Augustin*, 1957).

The present paper deals with adaptations of the original method to the measurement of antibodies (Ab) and to the assay of the very diluted Ag solutions required by the physician for prophylactic injection treatment against allergic complaints. Two plate methods will also be described, as well as methods for staining and preserving gel diffusion patterns for permanent record.

Materials

[illegible] vis Ltd.) [illegible] *ense* and [illegible] both the component extracts were used. These extracts have at least five major antigens in common, one of them a heat labile protein-carbohydrate complex. Pollen extracts prepared by extracting 1 g. of grass pollen with 10 ml. of buffered physiological saline (*Noon*, 1911) are said to contain 100,000 Noon units/ml. As this type of weight unit as well as definitions based on nitrogen or protein nitrogen estimations are generally considered unsatisfactory (*Augustin*, 1956), we indicate the potency of pollen extracts in terms of

* The authors wish to thank the asthma research council for a grant to the Institute in aid of this work

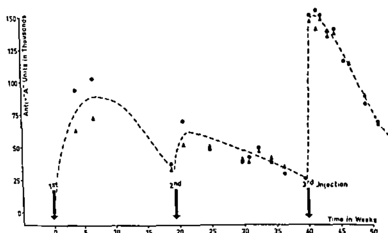


Fig 1 Level of antibody production in rabbits during one year, with only three (multiple) injections of the pollen proteins of *Dactylis glomerata* -- ▲ -- Gradient diffusion method, —●-- Precipitin method (optimal proportion method) Pooled sera of average titre.

the easily identifiable heat labile component which forms readily precipitating Ab in rabbits. In gel diffusion tests, the corresponding Ag Ab precipitation line is usually the first to appear and will be referred to as the "A" component. A fresh pollen extract prepared to contain 100,000 Noon units/ml. was used as a standard and was arbitrarily said to contain 100,000 "A" units/ml. Rabbit antisera to this component could then be described in the usual manner in terms of the corresponding "anti-A" units (optimal proportion method by *Dean* and *Webb*, 1926, and gel diffusion tests); this enabled us to prepare a freeze dried standard "anti-A" serum which became our primary standard for the assay of pollen extracts in terms of their "A" component

Antisera These were prepared in rabbits by multiple injections with antigen emulsions 400 mg. non-dialysable freeze dried residue of pollen extract was mixed with 10 ml of pollen extract containing 100,000 "A" units per ml. and 90 ml. 1% aluminium phosphate suspension (*Holt*, 1947). This was added to 100 ml. of 20% arlacel (Honeywell, Atlas Co. Ltd.) in Bayol F (Esso Petroleum Co. Ltd.).

n a hand emulsifier or

was obtained At one

ml. each were spread

(with the rabbit in the Trendelenburg position on the operator's lap) along the lateral aspects of the ventral surface of the animal,

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In 1955 *Augustin* and *Hayward* described a gradient double diffusion method in tubes for the assay of antigens (Ag). The theoretical aspects of this test together with a critical comparison with other gel diffusion techniques have been discussed in a previous paper (*Augustin*, 1957).

The present paper deals with adaptations of the original method to the measurement of antibodies (Ab) and to the assay of the very diluted Ag solutions required by the physician for prophylactic injection treatment against allergic complaints Two plate methods will also be described, as well as methods for staining and preserving gel diffusion patterns for permanent record.

Materials

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component extracts were used. These extracts have at least five major antigens in common, one of them a heat labile protein-carbohydrate complex. Pollen extracts prepared by extracting 1 g. of grass pollen with 10 ml. of buffered physiological saline (*Noon*, 1911) are said to contain 100,000 Noon units/ml As this type of weight unit as well as definitions based on nitrogen or protein nitrogen estimations are generally considered unsatisfactory (*Augustin*, 1956), we indicate the potency of pollen extracts in terms of

* The authors wish to thank the asthma research council for a grant to the Institute in aid of this work.

been used previously by *Oakley* and *Fulthorpe* (1953) in their simple double diffusion method, which was designed specifically for purposes of measuring Ab. (For the differences between the two methods cf *Augustin*, 1957.)

(2) *Direct assay by the reversed gradient diffusion method: Ag-gradient method.* This test has been developed similarly to the gradient diffusion method for measuring antigens (*Augustin* and *Hayward*, 1955), but, instead of an Ab-gradient across the middle layer, an Ag-gradient is established by incorporating Ag in the bottom gel layer, instead of Ab; Ab instead of Ag constitutes the top layer.

In practice, one part of a solution of Ag of a suitable concentration is warmed to about 55° C and mixed with three parts of 1% liquified agar of the same temperature. (Temperature requirements are not quite as critical as in the Ab-gradient – "direct" method –, and may be altered to suit individual requirements.) Quantities of about 0.5 ml. are dispensed (Pasteur pipette) into small tubes (diameter 0.8 cm., height 8 cm.) of equal bore to give agar columns of roughly equal heights. Care must be taken not to touch the sides of the tubes with the Ag-agar mixture After solidification each column of Ag-gel is overlayered with melted agar (0 5% 55° C). The height of this neutral column (containing neither reactant) is carefully adjusted by holding each tube against a reference tube during the filling process. A measuring aid may be used (*Augustin* and *Hayward*, 1955) but is not required after a little practice In this way, many tubes may be rapidly filled, all with columns of equal height. Any tube not conforming on subsequent checking is discarded. The height to be chosen for the middle layer depends on the Ag-concentration, the diffusion coefficients, time of incubation before adding the Ag, and on the required sensitivity, heights from 0.4–0.8 cm. are suitable. The tubes are closed with rubber bungs to prevent evaporation and placed in an incubator at 37° for a fixed period of time, usually 16 hours, for convenience After incubation, serial dilutions of antisera in isotonic saline are added to form the top layer A further period of incubation then follows. Precipitation lines may appear in a few hours, depending upon the concentration of the reactants Series of five tubes are set up for each antiserum under test together with a control set containing the standard antiserum A line forming tangentially to the upper meniscus is taken as the end point (fig. 2). If suitable concentrations have been chosen, the lines will maintain

eight of them as near as possible to the underlying lymph glands. This was followed immediately by an intraperitoneal injection of 5 ml., two intramuscular injections of 0.3 ml. each, and two further subcutaneous injections of 0.3 ml. each into the loose fatty tissue of the neck. Each rabbit received about 10 ml. of the emulsion or about 20 mg. grass pollen protein. A larger proportion of pollen protein should be included in the mixtures if Ab to components other than the main Ag are required.

Bleedings were taken from the ear vein, 50 ml. about every ten days, starting at roughly three weeks after the first injection. Fig. 1 shows that this method makes it possible to maintain high Ab-titres for at least one year, with only three sessions of multiple injections on one day every three months, and frequent bleedings. Single assays were carried out on each bleeding by the optimal proportion method of *Dean* and *Webb* (1926) and by the gradient double diffusion method (*Augustin* and *Hayward*, 1955).

Sera containing 0.02 % Na-azide as a preservative were either stored at 4° C, or freeze dried for storage at 4° C There was no deterioration on freeze drying and the titres of the two preparations with respect to a standard Ag-solution and in comparison with each other remained constant throughout the year.

Agar. 0.5 and 1 % solutions were prepared from Davis powdered New Zealand agar in hot isotonic saline. Clarification was carried out by filtration through filter paper pulp and was followed by steam sterilization. Clear colourless solutions were obtained. The bulk of the material was broken down into conveniently small quantities so that repeated melting of the agar was avoided as this causes breakdown and clouding. Sodium azide was added as a preservative to a concentration of 0.02 % directly before use.

Methods

The Assay of Antisera

(1) *Indirect method.* A standard tube is prepared such that a precipitation line forms just below the meniscus (gradient double diffusion method of *Augustin* and *Hayward*, 1955) when the upper layer contains a known amount of Ag mixed with a known volume of saline. By replacing the saline with serial dilutions of antisera, the antisera may be compared for their power to move the precipitation line into the meniscus. A somewhat similar procedure has

been used previously by *Oakley* and *Fulthorpe* (1953) in their simple double diffusion method, which was designed specifically for purposes of measuring Ab (For the differences between the two methods cf. *Augustin*, 1957.)

(2) *Direct assay by the reversed gradient diffusion method: Ag-gradient method.* This test has been developed similarly to the gradient diffusion method for measuring antigens (*Augustin* and *Hayward*, 1955), but, instead of an Ab-gradient across the middle layer, an Ag-gradient is established by incorporating Ag in the bottom gel layer, instead of Ab; Ab instead of Ag constitutes the top layer.

In practice, one part of a solution of Ag of a suitable concentration is warmed to about 55° C and mixed with three parts of 1% liquified agar of the same temperature. (Temperature requirements are not quite as critical as in the Ab-gradient – "direct" method –, and may be altered to suit individual requirements.) Quantities of about 0.5 ml. are dispensed (Pasteur pipette) into small tubes (diameter 0.8 cm., height 8 cm) of equal bore to give agar columns of roughly equal heights. Care must be taken not to touch the sides of the tubes with the Ag-agar mixture. After solidification each column of Ag-gel is overlayered with melted agar (0 5% 55° C). The height of this neutral column (containing neither reactant) is carefully adjusted by holding each tube against a reference tube during the filling process. A measuring aid may be used (*Augustin* and *Hayward*, 1955) but is not required after a little practice In this way, many tubes may be rapidly filled, all with columns of equal height. Any tube not conforming on subsequent checking is discarded. The height to be chosen for the middle layer depends on the Ag-concentration, the diffusion coefficients, time of incubation before adding the Ag, and on the required sensitivity; heights from 0.4–0 8 cm. are suitable. The tubes are closed with rubber bungs to prevent evaporation and placed in an incubator at 37° for a fixed period of time, usually 16 hours, for convenience. After incubation, serial dilutions of antisera in isotonic saline are added to form the top layer. A further period of incubation then follows Precipitation lines may appear in a few hours, depending upon the concentration of the reactants Series of five tubes are set up for each antiserum under test together with a control set containing the standard antiserum A line forming tangentially to the upper meniscus is taken as the end point (fig. 2). If suitable concentrations have been chosen, the lines will maintain

their positions indefinitely, as they do in our Ab-gradient diffusion method; differences in the Ab concentrations between consecutive tubes should be not less than 10%.



Fig. 2 [illegible]

middle tube (line at meniscus, indicator tube)

To obtain a stationary line in the meniscus, the Ag-concentration of the bottom layer had to be adjusted to 3,000 "A" units/ml. Ag-agar mixture, when employing a middle column of a height of 0.7 cm. (and a pre-incubation time of 16 hr.) This gave an end point of 1,000 anti-"A" units/ml. for the Ab in the top layer. These figures compare with 12,000 anti-"A" units/ml in the bottom layer and 800 "A" units/ml. in the top layer for a height of 0.7 cm. in the previous Ab-gradient diffusion method (*Augustin* et al., 1955).

There is an important difference between the Ab- (direct test) and the Ag- (reversed test) gradient diffusion methods since the AgAb precipitate is insoluble in excess Ab but soluble in excess Ag in the rabbit-type (precipitin) system. In both gradient double diffusion methods a gradient of the reactant in the bottom layer is established across the middle layer during the pre-incubation period. Immediately the other reactant is added Ag and Ab start to combine and a precipitate forms. As soon as precipitation starts there is increased diffusion of both reactants from their respective reservoirs in the top and bottom layers into the plane of reaction. In both tests, the reactant in the top layer is soon exhausted, leaving an excess of bottom reactant. In the Ab-gradient test, Ab is in excess, which cannot affect the AgAb precipitate (except by increasing its density). In the "reversed" test however, Ag is in excess, which may dissolve the precipitate. It is possible to get over this

difficulty by adjusting the Ag-concentration so that insufficient Ag is left in the bottom layer (after establishment of the gradient across the middle layer) to dissolve the precipitate once it is formed. If too high an Ag concentration is used, there is a steep gradient across the middle layer; precipitation lines may be seen to form shortly after addition of the Ab, but dissolve after further incubation, thus invalidating the advantages of the gradient diffusion test over the simple double diffusion method of *Oakley* and *Fulthorpe*. Great care must be taken therefore to choose an Ag concentration sufficiently high for the precipitation line to form in the meniscus, but low enough not to dissolve the precipitate. Once the correct Ag concentration in the bottom layer is found (in relation to the length of the middle layer, the time of pre-incubation and the Ab concentration aimed at in the indicator tube) the test can be carried out exactly as the Ab-gradient test. Similar considerations apply to the adaptation of the gradient diffusion method to "horse" systems where the precipitate is soluble in excess Ab as well as in excess Ag.

The Dilute Antigen Test

This test is designed to check (rather than assay) the potency of pollen extracts containing 100–200 "A" units per ml. Such solutions have a total nitrogen content of 1–2 μg./ml of which about one-fifth is protein nitrogen, only a fraction of which represents the Ag we assay. The required test must therefore be sensitive enough to detect such small quantities and accurate enough to distinguish between two such quantities. Since the smallest quantity measurable by the Ab-gradient diffusion method is 800–1,000 "A" units/ml, some type of blending method has to be employed. The least laborious and most accurate method for this purpose was found to be an application of the gradient diffusion method as an "indirect reversed" test

The diluted Ag-solutions are added to a series of standard antisera and incubated for 30 min. Ag and Ab combine, thus reducing the Ab concentration of these antisera (indirect measurement of Ag). These reduced concentrations are then measured by the reversed test

Fig 3 shows schematically how a suitable set of tubes may be prepared for checking an unknown solution suspected of containing 100 or 200 "A" units/ml. The Ab-gradient across the middle layer

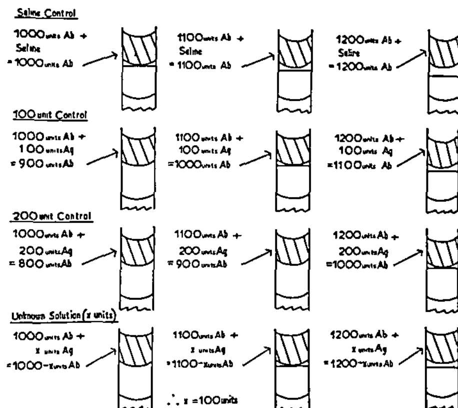


Fig 3 Dilute Antigen Test "Reversed" method, Ag incorporated in gel in bottom layer

is established in the usual manner. For the top layer 0.05 ml. (1 part) of antiserum is mixed with 0.45 ml. (9 parts) of the Ag-solution, i.e. dilutions of the Ag solutions are kept at a minimum. The three control tubes (top series in fig. 3) contain saline in place of Ag and concentrations are arranged so that a line forms in each tube. The first tube (fig. 3) represents the indicator position. The addition of 100 "A" units to each top layer (in place of saline) makes the middle tube the indicator tube (second row in fig. 3), i.e. lines are being formed in only two tubes. When 200 units of Ag are added (third row, fig. 3) a line remains in only one tube These delicate comparisons can readily be achieved in a set of only 9 tubes using 1.5 ml. of the solution under test

A similar "Nine tube arrangement" can be devised using the usual Ab-gradient method, the control series is made to contain 800, 900 and 1000 units of Ag/ml., the 1000 units/ml. tube only showing a line (touching the meniscus). The addition of the 100 and 200 units solutions in place of saline would then cause the

appearance of lines where there were none in the control tubes ("negative control"). In practice, it is however more satisfactory to have a control series showing lines ("positive control") which are made to disappear by adding the unknown (weak) Ag solutions to the top layer.

Plate Methods

(1) *Rapid plate method* This is a single diffusion method of which a preliminary account has already been given (*Augustin*, 1956). One part Ab (or Ag) of a suitable concentration is heated to 54° C and mixed with 3 parts of a 1% agar solution cooled to 54° C. This mixture is poured into Petri dishes or on to glass sheets to a depth of about 3 mm. When set, circular wells, 0.2 cm. in diameter, are cut into the gel at distances of about 1.5 cm. from each other. After filling the wells with serial dilutions of Ag (or Ab) the gel plates are placed in air tight containers and incubated at 37° C. With Ab incorporated in the gel, rings of precipitate appear around the wells while haloes of precipitate appear when Ag is the reactant incorporated in the gel (as would be expected since AgAb precipitate is soluble in excess Ag, but insoluble in excess Ab).

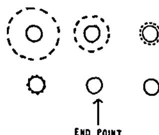


Fig 4 Diagram of simple diffusion plate assay Antiserum incorporated in the gel Cups filled with serial dilutions of antigen

The diameters of the precipitation rings or haloes decrease with decreasing concentration of the reactants in the well until precipitation finally occurs in the well instead of in the gel. The first well not to be surrounded by a ring or halo is taken as the end point (fig 4) and the dilution at which this occurs in comparison with a standard may be used to measure the concentrations of the Ag or Ab solution

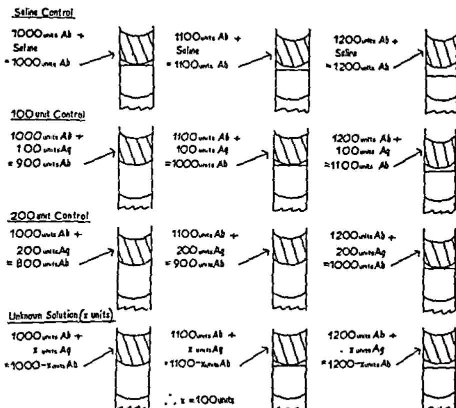


Fig. 3 Dilute Antigen Test "Reversed" method, Ag incorporated in gel in bottom layer

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The peripheral wells are filled with the reactants under investigation (say Ag), the unknown and the standard being placed in alternate wells. The central well contains the other reactant (say Ab). Precipitation lines normally appear within 16 hr. If unknown

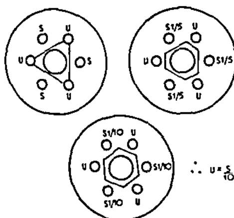


Fig 5 The Polygon plate method Diagrammatic representation Central wells contain Ab Peripheral cups contain Ag S is the standard Ag solution, U is the unknown Ag solution

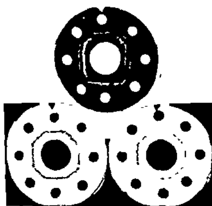


Fig 6 The Polygon plate method Each hole punched separately by hand Central wells contain Ab Peripheral wells contain standard Ag alternating with a dilute unknown Ag The standard solution was diluted until a shape approaching a regular polygon was obtained

and standard are of very different concentrations a triangle or square will be formed by the stronger solution in place of a hexagon or octagon. On diluting the stronger reactant irregular polygons form (fig. 6) until the formation of a regular polygon indicates

5,000 anti-"A" units/ml. gel was found to be the lowest concentration at which easily discernible precipitation rings were formed. At this Ab concentration, the end point for Pollacine was 2,000–3,000 "A" units/ml. i.e. three times as high as in the gradient diffusion method. Differences in Ag concentrations between consecutive wells had to be of the order of 25 % to give a clearly cut end point. With Ag incorporated in the gel similar limiting figures were obtained.

If Ag and Ab concentrations are sufficiently high, precipitation rings (or haloes) may form within the first hour of incubation, but at a level of 5,000 anti-"A" units/ml. several hours are required.

As this test is basically a single diffusion method it is bound to be less sensitive than the gradient diffusion tests; it is also less accurate, but has proved very useful in laboratory work where quick results are required.

(2) *The Polygon plate method.* This test was developed accidentally when comparing Cocksfoot pollen extracts with their ultra-filtrates. A small amount of skin reactivity (0.1 % of the original) remained in these ultra filtrates and the question arose whether there was a general small leakage through the membranes of a number of Ag molecules of different sizes, or whether there was some active molecule, smaller than the rest, that could leak through selectively. Three Ouchterlony plates were set up similarly to fig. 5; the central well was filled with anti-serum (rabbit) to cocksfoot pollen extract and the ultra-filtrates and concentrates (reconstituted to their volume before ultra-filtration), were placed in alternate wells around the periphery. At first, it appeared that the filtrate did not contain any detectable Ag. However, by diluting the concentrate one-thirtieth, a regular polygon was formed and all the original antigenic components were found to be present in the ultra-filtrates as well as in the concentrates. Selective leakage was therefore judged to have been unlikely.

Fig. 5 shows diagrammatically the quantitative application of this test. Once more gel slabs of about 0.3 cm. thickness are prepared with central wells of about 0.5 cm. diameter. Six or eight wells, about 0.2 cm. in diameter are symmetrically spaced around the central well to give regular hexagons or octagons. The distance from the centre of the large well to the centres of the small wells may be varied to suit the concentrations of the system under investigation and the degree of resolution (of a complex system) required.

cerol content as suggested to one of us (R.A.) by *Uriel**; i.e. it is necessary to experiment with each type of gel to find the correct concentration of glycerol, which depends on the degree of breakdown of the gel. It is possible to preserve the gel film – which becomes somewhat cloudy and a little brittle on drying – without the incorporation of glycerol, by coating with a lacquer. *Gell* (1955) has used Bedacryl (Bedacryl 122X, I.C.I. Ltd., England) for this purpose; we found Ercalene (Canning Ercalene, Grade A, Cat. No. 1461, Birmingham, England) more convenient to use (e.g. less viscous, a less noxious solvent). When water is carefully excluded, gel layers thus coated are transparent and precipitation lines show up clearly, whether stained or unstained.

Gel films left to dry on glass and lacquered as mentioned, may be used directly as lantern slides (without intervening photography). Gel films dried on sheets of glass adhere tenaciously to the glass surface, but wet gel slabs float off easily in water or saline. In this state they may be transferred on to sheets of cellophane and allowed to dry, with the wet cellophane stretched over a beaker, etc.; more conveniently, the gel slabs may be transferred to sheets of glass coated with silicone (e.g. by treating with "Repelcote" Hopkins, Williams Ltd., England) i.e. to a surface to which they cannot adhere. In both instances, paper thin gel films are eventually obtained which may be preserved indefinitely after covering with one or two coats of Ercalene. If the gel slab contains a suitable amount of glycerol, coating is unneccessary

Method

(a) Washing. The fully developed Ouchterlony plates are immersed in isotonic saline (0.85% NaCl) and washed for a minimum of three hours – preferably overnight – *with at least three* changes of saline. During this process, the gel slabs (marked by means of notches or with a suitable dye) *float free*. They are then placed in a gentle stream of running tap water for a further washing of about five hours.

(b) Drying. The washed gel slabs are transferred to fresh glass sheets (silicone coated, or uncoated lantern slides), care being taken to avoid the formation of air bubbles between gel and glass.

* We wish to express our thanks to Dr *Uriel* for this suggestion.

equal concentrations of unknown and standard. With complex systems, several regular and irregular polygons may form and may be adjusted in turn with respect to the components systems.

This test is not as sensitive as the tube test, but is more sensitive than the rapid plate method. 25% differences between the concentrations of unknown and standard result in the formation of polygons of sides sufficiently different to be obvious to the naked eye.

The polygon method also helps in the qualitative interpretation of Ouchterlony tests in general where the establishment of balanced conditions is so important for correct interpretation: Only then can sharp stationary lines be expected to form. In the polygon method balanced conditions may be obtained in turn for all the component systems. Unsuspected common constituents may thus be uncovered.

Another useful type of polygon method has since been described by *Gell* (1957). He places serial dilutions of Ag solutions around standard and unknown antisera and looks for the formation of similar regular polygons. Precipitation lines of decreasing intensity are formed, disappearing where the solutions are very dilute. The two polygon methods are complementary.

Staining of protein patterns in agar and their preservation as permanent records. Staining methods for proteins and other compounds have been widely used in connection with paper chromatography and paper electrophoresis. Recently similar methods have also been employed for staining protein precipitates in agar. So far aqueous dye solution have been used (*Uriel* and *Scheidegger*, *Uriel* and *Grabar*). We prefer solutions of Bromophenol blue in ethanol, because staining can be carried out more quickly in ethanol and background colour washes out readily without affecting the strongly stained protein "lines".

Staining may be carried out on the wet (washed) agar slabs (*Bjorklund*, 1954), or after drying the 3–5 mm. thickness of the gel to a thin film – as has been done by *Uriel* and *Scheidegger* (1955) and *Uriel* and *Grabar* (1955) By incorporating 15% glycerol in the gel, the latter workers were able to obtain a pliable film that could be detached from the glass. However, with the agar used in our laboratory, films of a sticky consistency were obtained which were quite unusable. The difficulty was overcome by reducing the gly-

2) Two semi-quantitative gel plate methods are described for assaying antigens and antibodies.

3) Methods are given for staining and preserving antigen-antibody gel diffusion patterns.

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A wad consisting of three or four sheets of wet filter papers is then carefully laid on the surface of the gel and gently pressed down, again taking care to avoid the formation of air bubbles; excess moisture is then soaked up from the surface of the pad by means of a thick absorbent cloth. This is followed by drying at about 37°. The filter paper pad is essential for even drying of the gel slab. When dry, the thin gel film may be removed readily (from the silicone treated glass) by gently prising it from the glass with the help of a razor blade. If the filter paper has not completely peeled off during the drying, any remaining bits will soften in the subsequent immersion in liquid and can be removed by gently rubbing with the finger tips.

(c) Incorporation of ethanol and/or glycerol into the gel film. The dry gel film (by itself or stuck to glass – again, the film will not float free if it is dried while adhering to uncoated glass), is placed into a series of baths, all containing 8% $^{v}/_{v}$ glycerol and increasing amounts of ethanol; forty minutes are allowed in 50% ethanol, forty minutes in 70% and forty minutes in 90% ethanol. Ten minutes in each of the ethanol concentrations is sufficient if glycerol is omitted (the omission of glycerol necessitates coating with a lacquer). After soaking in 90% ethanol (with or without glycerol) the gel slabs are ready for staining

(d) Staining Two to three minutes in 0 1% $^{v}/_{v}$ bromophenol blue is usually sufficient; the exact time required is readily judged after a little practice; the protein lines must appear dark without overstaining the background. Excess dye is removed by washing in several changes of 90% ethanol (with or without the inclusion of 8% glycerol, depending on whether lacquering is ultimately required, e.g. for the preparation of lantern slides) – a few min. in each until the background is free from dye. Gel films are then placed face downwards on the silicone coated glass without removing excess moisture). This is followed by drying at 37°. The dry films are once more prised gently off the silicone treated glass and are pliant enough to be filed without further treatment.

Summary

1) Adaptations of the original gradient gel diffusion method (*Augustin* and *Hayward*, 1955) are given for the assay of antibodies and quantities of antigens of <1 µg./ml.

disease, the antigen being autologous – namely, the unchanged blood-cell. In the second case, there is no true autosensitization because antibodies are formed against blood corpuscles which are altered by exogenous factors.

As is well known, tissue specific antibodies have been demonstrated in the course of some chronic diseases such as glomerulonephritis, rheumatic carditis, encephalomyelitis, hepatitis etc. Obviously tissue-auto-antibodies could only be shown in such cases when the disease was already manifest. Therefore, it has been felt that antibody formation cannot take place against normal, unaltered autologous tissues. It is significant in this connection that autoantibodies have been demonstrated above all in cases of inflammatory organic diseases of bacterial or viral origin. In such cases it can be assumed that endogenous hapten-like substances combine with certain bacterial products (lipids) to form a complete antigen. Another possibility would be that the tissue of the organ will be destroyed or altered considerably under the influence of the inflammation and become antigenic. The question whether the course of a primary infectious organ disease will be influenced by the production and the effect of organ specific antibodies is still open to discussion

Experimental results seem to allow a more positive attitude towards the problem of the pathogenic importance of organ specific antibodies The results of *Freund* are of special importance. According to them it is possible to produce encephalomyelitis, neuritis, uveitis and aspermatogenesis in rabbits, guinea-pigs or in other animals, by injecting autologous or homologous tissue suspensions combined with the complete adjuvant of *Freund* (paraffin oil and killed mycobacteria), the animal diseases so produced are comparable with some human diseases *Lumsden*, *Collins*, *Waksman* et al. reported on similar results. Neither the injection of tissue suspensions alone nor the application of the adjuvant only are able to induce tissue changes of this kind

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The killed mycobacteria in the adjuvant enhance the immunologic response of the organism: antibody production seems to be

Dornbusch, S. · Int Arch Allergy *11* 206—213 (1957)

The Value of the Gel-Precipitation Method for the Study of Auto-Immunological Problems

By SIGRID DORNBUSCH

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In recent years it has repeatedly been demanded "to relegate the term 'horror autotoxicus' once and for all to the shelves of medical history" *(Edsall)*. Actually, *Ehrlich* wanted to state by this term that according to our practical knowledge, antibody formation against autologous or homologous protein is impossible.

It is doubtful, however, whether the latest clinical and experimental experiences will sufficiently justify this principal attitude Some authors (e.g., *F. F. Krusius*, *P. Uhlenhuth* and *Handel*, *Andrejew*, *G. Kapsenberg*) reported more than fourty years ago that in experiments on animals, antibodies can be produced by injecting autologous or homologous material In most cases, however, organ extracts were used which had been specially prepared for injection and which can hardly stand comparison with endogenous substances.

The existence of an auto-allergic pathogenesis was taken into consideration first when – in connection with various hematologic diseases – antibodies could be detected against autologous erythrocytes and leucocytes respectively. Up to the present time, it has been impossible, however, to state with certainty whether such antibody-production is suddenly started against unaltered blood-corpuscles due to an individual allergic disposition of the organism or if, under the influence of exogenous factors (bacterial products, drugs, etc.) the surface of blood corpuscles becomes changed or "charged" and thereby acquires antigenic properties. The first alternative only would fulfil the definition of a true auto-allergic

to form a complete antigen. It is doubtful, though, whether antibodies against such a combined antigen can also react with the unaltered tissue. In animal experiments no tissue changes could be produced if instead of killed mycobacteria only certain lipids of the bacteria were used in the adjuvant. The results do not permit so far to decide whether autosensitization depends mainly on a certain reaction in the organism or, rather, on the presence of certain bacterial substances. If auto-sensitization depends mainly on a certain mode of reaction of the organism, it should be possible to prove the existence of tissue specific antibodies in connection with abacterial tissue changes. If for instance by earlier contact with tubercle bacilli the conditions necessary for a certain type of reaction have already been supplied, the possibility may exist that when a disintegration of tissue starts and cell substances will reach the sites of antibody formation – antibodies will be produced against these substances. The solution of this problem would be of the utmost importance for any further discussion of auto-allergic diseases

We examined the sera of more than 1000 patients by means of the highly sensitive hemagglutination test according to *Boyden* and found that even in cases with degenerative diseases without any bacterial influence, tissue specific antibodies may be traceable (*Kleinsorge*, *Dornbusch*). For various reasons these investigations were carried out chiefly in cases with necrotic changes of the myocardium The present report will be confined to the demonstration of antibodies directed against cardiac tissue.

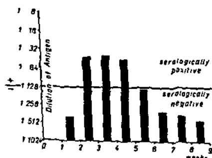


Fig 1 Average value of the auto-antibody-titer of 15 patients in *the first 9* weeks after recent myocardial infarction

possible even against substances which are usually not or only weakly antigenic, such as autologous protein. In other words: the slight immunologic differences by which injected autologous or homologous material can be distinguished from altered endogenous material will be enhanced by the adjuvant and the organism produces antibodies. These are at first directed against the injected altered tissue material, but at the same time they have obviously an affinity to the corresponding tissues of the injected animal. As stated above paraffin oil enhances the production of antibodies and it may be that that amount of antibodies which cannot be "neutralized" by the injected tissue antigen, reacts with the corresponding tissue of the injected animal, and causes tissue damage. Quite a few observations substantiate this as yet, so for instance the fact that injection of a suspension of brain-substance does not induce any damage to peripheral nerves, while neuritis can be produced by the injection of peripheral nerve tissue.

The existence of auto-allergic experimental diseases is sufficiently proved. The question, however, still remains unsolved, whether similar autoimmunologic mechanism play a role in human diseases. The demonstration of organ specific antibodies in some disease states does not prove that these antibodies elicit the specific tissue changes.

As stressed above, the existence of organ specific antibodies has been revealed above all in connection with bacterial inflammations. It may therefore be supposed that the bacteria exert an effect similar to that of the adjuvant. Thus it seems to be possible that together with the defense mechanism against bacterial invasion, a reaction against endogenous substances comes about. Such substances may be transferred from the damaged tissue into the circulation and could then, just as injected autologous or homologous tissue suspension in animal experiments, elicit the production of antibodies. When the antibody concentration exceeds the humoral antigen concentration a reaction with tissue-cells in the corresponding organs may take place. Attempts have been made to explain the gradual transition of an acute inflammation into a chronic one by such a mechanism.

Another view is that bacteria take an active part in the development of an auto-antigen. According to this view, endogenous substances – possibly leaving the cell during an inflammatory damage of the tissue – may combine with some bacterial substance

to form a complete antigen. It is doubtful, though, whether antibodies against such a combined antigen can also react with the unaltered tissue. In animal experiments no tissue changes could be produced if instead of killed mycobacteria only certain lipids of the bacteria were used in the adjuvant. The results do not permit so far to decide whether autosensitization depends mainly on a certain *reaction in the organism or, rather, on the presence of certain* bacterial substances. If auto-sensitization depends mainly on a certain mode of reaction of the organism, it should be possible to prove the existence of tissue specific antibodies in connection with abacterial tissue changes If for instance by earlier contact with tubercle bacilli the conditions necessary for a certain type of reaction have already been supplied, the possibility may exist that when a disintegration of tissue starts and cell substances will reach the sites of antibody formation – antibodies will be produced against these substances. The solution of this problem would be of the utmost importance for any further discussion of auto-allergic diseases

We examined the sera of more than 1000 patients by means of the highly sensitive hemagglutination test according to *Boyden* and found that even in cases with degenerative diseases without any bacterial influence, tissue specific antibodies may be traceable (*Kleinsorge, Dornbusch*). For various reasons these investigations were carried out chiefly in cases with necrotic changes of the myocardium The present report will be confined *to the demonstration* of antibodies directed against cardiac tissue.

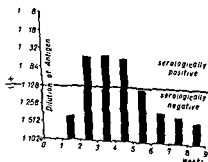


Fig 1 Average value of the auto-antibody-titer of 15 patients in the first 9 weeks after recent myocardial infarction

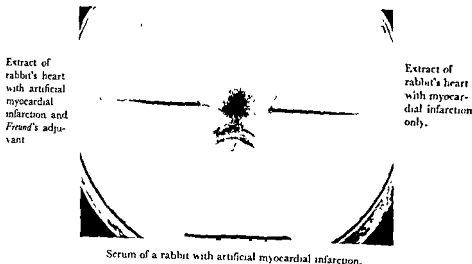
Of 46 patients with clinical diagnosis of an infarction of the myocardium, only 17 i.e. 34,2 % had antibodies against homologous cardiac tissue. Fig. 1 shows that these tissue antibodies appear at certain intervals only, and apparently in low concentration. (Quantitative evaluation was performed according to a modification of the *Boyden* method suggested by *Vorlænder;* the results are to be taken relatively.) Direct relations of any kind between the appearance of antibodies to heart tissue, and the character and course of the infarction of myocardium were not observed.

Even more striking is the fact that only in the minority of the cases examined could homologous antibodies to myocardium be found, whereas in cases with inflammatory diseases – e.g., glomerulonephritis – homologous antibodies to the affected tissue can be demonstrated nearly regularly *(Vorlænder).* We did not succeed, however, in connecting the antibody formation to heart tissue with a peculiar allergic disposition. A coincident bacterial infection could also altogether be excluded. It is remarkable, however, that the erythrocytes treated with tannic acid and an extract from normal human cardiac tissue had been agglutinated by sera of patients with inflammatory rheumatic myocarditis as well as by sera of patients with an obviously aseptic infarction of the myocardium. This observation may well substantiate that the same kind of antibodies are produced independently whether the tissue-damage was caused by bacterial influence or by a degenerative process The hemagglutination method, however, does not allow to differentiate between tissue-antibodies formed under various conditions. By adsorption-tests the presence of antibodies against tissue lipoids could be excluded. By application of more specific but less sensitive methods, however, the very small quantity of antibodies could not be demonstrated.

In order to obtain information about the significance of our findings, we planned a series of animal experiments, based on the following working hypothesis: if the adjuvant, resp. the bacterial infection exerts only a potentiating influence upon the development of an auto-allergy it should be possible to induce the production of tissue-antibodies by injecting *Freund*'s adjuvant alone into animals with aseptic degenerative changes of certain organs. We therefore produced infarctions of the myocardium in rabbits by injecting of adrenalin accordingly to *Ichteimann.* Unexpectedly, in the serum of some animals (about 4 %) we found tissue-antibodies

already after the pretreatment with adrenaline. The antibodies reacted in the *Boyden*-test in a specific manner with the erythrocytes coated with an extract of rabbits' hearts *(Dornbusch)*. These antibodies were present only during a short period and could not be detected by any other immunologic method.

Fig 2 Ouchterlony-Plate



We then injected, analogous to *Cavelti*'s experimental arrangement, simultaneously with injections of adrenalin, streptococci or streptococcal toxin into a series of animals. No essential differences could be discerned in comparison with the experimental series which had been subjected to a treatment with adrenalin only. Another series of animals pretreated with adrenaline was injected several times intraperitoneally with *Freund*'s adjuvant. In the *Boyden*-test with rabbit-heart-extract coated erythrocytes as antigen, the serum of these animals produced a much stronger effect and over a longer period, compared with the above mentioned group. Moreover, the sera of these animals gave precipitation lines in the gel-diffusion test (according to *Ouchterlony*) if they were allowed to diffuse against an extract of rabbits' hearts. In ...

... antigenic substances. ... the differentiation

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Discussion

On Freund's Adjuvants

Dr *Colover** stated that he had been working on experimental allergic encephalomyelitis and had certain points which he would like to make about the Freund adjuvants. The residues obtained from acid-fast bacilli could be substituted for the bacilli and intact bacilli were not essential Some of the lipid extracts made from the bacteria showed only partial activity, which required special methods to demonstrate

Regarding the brain factor in the production of experimental allergic encephalitis, it was possible to substitute protein made from brain for the whole brain and this protein could be used in much smaller amounts than the intact brain – about 1/250th of the amount of wet brain required (1)

He also wished to draw attention to the importance of the actual site of inoculation of the material into animals as this might considerably affect the results obtained and it was possible to inject materials into one site and produce little or no reaction *in the nervous* system and yet when they were injected elsewhere quite severe lesions would appear in the brain He did not consider that the Freund adjuvant type of material need necessarily be derived from bacterial sources as it was quite conceivable tha[illegible]

[illegible] simple saline extract of an organ such as had been described might not be an adequate antigen for this purpose

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* Canadian Red Cross Memorial Hospital, *Taplow*, Berks (England)

With the gel-diffusion-method we also could show (Fig. 2) that a marked difference exists between the adjuvant-antigen-antibody complex and the complex of tissue antigen-antibody. This can be perhaps explained by assuming that some component of the adjuvant (mycobacteria or protein) forms a new antigen together with a tissue factor. *Freund*'s view on the adjuvant's mode of action is essentially supported by these results, without receiving conclusive proof. We hope to be able to say a final word in this subject in the near future by combining the gel-precipitation method with gel-electrophoresis. At present we can only assume that the antibodies demonstrated in the hemagglutination test after artificial injury to the myocardium are identical with those antibodies which form precipitation lines with an extract from rabbits' hearts.

Our original problem as to the possibility of antibody formation against endogenous protein may already now be answered in the affirmative. The most important pre-requisite for auto-antibody production is presumably an injury to the tissue which renders it possible that substances which normally do not enter the circulation, come into contact with the sites of antibody production.

Summary

In our earlier experiments it was found, that even in cases with degenerative diseases without any bacterial influence tissue-specific antibodies may be traceable, but only by means of the highly sensitive hemagglutination test according to *Boyden*. As this method does not allow to differentiate between tissue-antibodies formed under various conditions, we tried to demonstrate these auto-antibodies with the help of the gel-precipitation method. This was possible after having enhanced the antibody-concentration by injections of *Freund*'s adjuvant The adjuvant's mode of action in processes of auto-immunisation and the favourable conditions for further research concerning the differentiation of auto-antigenic substances by the gel-precipitation method are discussed.

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transient symptoms The question arises as to whether only the individuals of the former group can claim to possess a definitely allergic constitution. The following items may be classed as symptoms of an allergic constitution («allergic stigmata»): a history of allergic disease in the family of the patient, a personal history of other forms of allergic disease, the typical histological findings, eosinophilia of the blood and secretions, hypotension, changes in the blood sugar curve (which often continues at a very low level), increased permeability of the capillary walls, frequent slight changes in the electro-encephalogram, changes in the blood cholesterol, the potassium content of the blood and the protein pattern, often a slight increase in basal metabolism and diminished values obtained in adrenal function tests (tables I and II).

Table I

Allergic Stigmata in Patients with Atopic Dermatitis

I	Positive family-anamnesis	76% (100 cases)
II	Positive auto-anamnesis	65% (100 cases)
III	Blood-eosinophilia (many patients with high blood percentage of eosinophils)	58% (100 cases)
IV	B S R decreased	34% (100 cases)
	increased	19% (100 cases)
V	Low blood sugar curve	33% (100 cases)
VI	No, or low, free gastric acid	70% (50 cases)
VII	Low blood pressure	56% (100 cases)
VIII	Positive skin tests	81% (100 cases)
IX	Increased permeability Cap wall	70% (100 cases)
X	B M above normal	29% (100 cases)
	under normal	1% (100 cases)
XI	Decreased 17-ketosteroids / 24 hours	27% (59 cases)
XII	Increased percentage of γ-globulin	80% (50 cases)

In only 2 cases examined we did not find any stigmata, and in 2 other cases only 2

Table II

Allergic Dermatitis in Patients with Allergic Respiratory Diseases

1000 asthmatic patients	Eczema infantum	150 (15 %)	(41,2%)
	atopic dermatitis	206 (20,6%)	
	eczema infantum + dermatitis	56 (5,6%)	
	no eczema	588 (58,8%)	
	(alternation of asthma and eczema, 12 cases)		
500 rhinitis patients	eczema infantum	24 (4,8%)	(32,0%)
	atopic dermatitis	120 (24 %)	
	eczema infantum + dermatitis	16 (3,2%)	
	no eczema	340 (68 %)	

Quarles van Ufford, W. J. Int. Arch. Allergy 11, 214—217 (1957)

Histamine Provocation and Histamine Treatment of Atopic Dermatitis

W. J. QUARLES VAN UFFORD

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(Director *M. A. van Melle*, M. D.)

Should all children who have had an occasional attack of urticaria after eating an egg or strawberries be called «allergic children»? These symptoms only persist or increase in a small number of individuals. When a new kind of wood from Suriname was used in a factory in Utrecht, 60 to 70 workers responded to contact with this wood by developing contact dermatitis. Work was carried on as usual, but a few months later, the factory physician found that the symptoms had disappeared in the great majority of cases. Only one worker continued to show severe symptoms and he was transferred to a different department, which resulted in the rapid disappearance of his symptoms (elimination therapy with a contact allergen in a patient with atopic dermatitis?)

During their apprenticeship, many florists go through a period marked by skin reactions following the unaccustomed contact with flowers. Only a few continue to show symptoms and either have to change their occupation or go on working despite the eczema. During their period as probationers, many nurses develop skin reactions as a result of the unaccustomed contact with penicillin, streptomycin, procaine or vitamin B, though the symptoms persist in only a small number of individuals who either have to stop working or have at least to be treated with special care. It is open to doubt whether there is any fundamental difference between patients whose symptoms persist and the other group, in which we were interested, which merely showed

We usually started with one treatment daily (the dosage depending on the response of the patient), which is changed to treatment 3 times weekly after about 7 days and subsequently reduced to once or twice weekly for a few weeks.

The results of treatment varied markedly. In some cases, the symptoms disappeared completely In that event, treatment was discontinued and again repeated when the symptoms recurred. In a number of cases, only the itching was relieved, whereas the skin symptoms persisted or were suppressed only in part. In a third group, no improvement or merely very inadequate improvement was obtained (table IV) The difficulty in observing the results of a therapy of itching is that many psychological factors are involved in this, and that in general atopic dermatitis suddenly comes and again suddenly almost disappears Especially for treatment with histamine by iontophoresis we had to expect a strong suggestive support. The use of histamine administered by iontophoresis as a provocation test in atopic dermatitis may be regarded as a valuable addition to the methods of examination available, and as a therapeutic agent should certainly be borne in mind in difficult cases. In some cases, it is possible to observe how the histamine sensitivity decreases

Summary

In many cases of atopic dermatitis, different so-called allergic stigmata were found As several times the specific allergens could not be found, a provocation test with histamine by iontophoresis seemed useful The same method used as treatment was rather disappointing the itching reacted favourably (somatic or psychological treatment)?

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When we see a patient with a dermatitis and name it an allergic dermatitis, we have to try to give a specific treatment. As in practice we do not always find the allergens, or still more difficult, the complex of allergens or factors, we must look for other treatments.

In patients in whom a sufficiently specific therapy (elimination etc.) was frequently impracticable until they had been observed over a prolonged period, we often started especially in cases of a dermatitis with an infection, with an auxiliary treatment based on injections of histamine – staphylococcus toxoid – staphylococcus vaccine.

In our attempts to detect allergic stigmata and devise methods of provocation-tests, we also resorted to a provocation test with histamine (administration of histamine by iontophoresis, using a 1.0% solution of histamine acid phosphate). In evaluating the response, we differentiated between very marked general histamine sensitivity reactions and aggravation of the actual symptoms (i. e. of the eczema) following administration of histamine even after administration of a single dose in some cases, following administration of several doses in others (table III). Only the last reaction, however, is to be considered as a real provocation test.

In conclusion, a number of patients were treated with histamine by iontophoresis

1. to relieve the very often intense itching,
2. to relieve the skin symptoms themselves.

Table III

Histamine (Iontophoresis) Provocation Test in Patients with Atopic Dermatitis

100 cases (control tests negative)	
very high histamine sensitivity (after 2–4 minutes already headache, general itching, flush, vertigo, etc.)	20 (20%)
Reaction in the dermatitis after 1 provocation	20 (20%)
Reaction in the dermatitis after more provocations	50 (50%)
No reaction	30 (30%)

Table IV

Treatment of Atopic Dermatitis Patients with Histamine by Iontophoresis

50 cases (control cases negative)

I. Temporary improvement of itching 40 cases (80%)
II. Improvement of dermatitis· 20 cases (40%)
III No improvement of dermatitis. 30 cases (60%)

We usually started with one treatment daily (the dosage depending on the response of the patient), which is changed to treatment 3 times weekly after about 7 days and subsequently reduced to once or twice weekly for a few weeks.

The results of treatment varied markedly. In some cases, the symptoms disappeared completely. In that event, treatment was discontinued and again repeated when the symptoms recurred. In a number of cases, only the itching was relieved, whereas the skin symptoms persisted or were suppressed only in part. In a third group, no improvement or merely very inadequate improvement was obtained (table IV). The difficulty in observing the results of a therapy of itching is that many psychological factors are involved in this, and that in general atopic dermatitis suddenly comes and again suddenly almost disappears. Especially for treatment with histamine by iontophoresis we had to expect a strong suggestive support. The use of histamine administered by iontophoresis as a provocation test in atopic dermatitis may be regarded as a valuable addition to the methods of examination available, and as a therapeutic agent should certainly be borne in mind in difficult cases. In some cases, it is possible to observe how the histamine sensitivity decreases.

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In many cases of atopic dermatitis, different so-called allergic stigmata were found. As several times the specific allergens could not be found, a provocation test with histamine by iontophoresis seemed useful. The same method used as treatment was rather disappointing: the itching reacted favourably (somatic or psychological treatment)?

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Augustin, R Int Arch Allergy *11* 218–222 (1957)

Pollen Antigens versus Pollen Allergens. Limiting Aspects of Gel-Diffusion Methods for Assay Purposes in Allergy

By R. Augustin*

From the Wright-Fleming Institute, St Mary's Hospital Medical School, London

Recently, much work has been expended on assaying pollen and other allergenic extracts by means of gel diffusion methods, in terms of constituents antigenic in rabbits (7, 11, 4).

Are we justified in doing this? Are there "rabbit antigens" identical with the substances that are antigenic in man, i.e., the substances reponsible for the formation of reagins?

Certainly, we have as yet no means of telling whether the substances responsible for forming antibodies in the rabbit are identical with those responsible for forming reagins in man. In fact, all our attempts to inhibit the precipitation of rabbit antibodies with pollen antigens by means of the appropriate reaginic sera (added to the antigen prior to the addition of the rabbit antisera) have failed so far. This does not necessarily mean that different antigens are involved in the two reactions. It may merely indicate that precipitating (rabbit) antibodies are more "avid" for the pollen antigens than the reagins, i.e that the reagin-pollen antigen combination easily dissociates – or that some additional factor is required for this combination to take place at all. So far, we have not been able to find such a hypothetical additional factor, although we have experimented with a large number of human and animal tissues and body fluids

However, we do know that any hypothetical allergens cannot easily be dissociated from the protein complexes antigenic in rabbits; so far we have been unsuccessful in doing this by a large number of chemical fractionation procedures and chromatography

* Thanks are expressed to the Asthma Research Council for a grant to this Institute in aid of this work.

(2, 3). Indeed, pollen antigen complexes with rabbit antibodies may be washed many times (up to twelve times washing with cold saline in a recent experiment in our laboratory), and yet will still give positive skin responses in allergic subjects when the antigen-antibody floccules are dissolved in dilute alkali. From this alone it would appear that there are at least some skin reactive components which are antigenic in both rabbit aud man. On the other hand, the possibility remains that substances allergenic in man (and skin reactive in allergic subjects) adhere closely to the pollen components precipitated by the rabbit antibodies.

We do know that pollen allergens are closely associated with the pigmented protein-carbohydrate complexes that are antigenic in rabbits, and that the protein part alone is involved in allergenicity (3), since proteolytic digestion with three different crystalline enzymes, active at pH 1.5, 6 9 and 8 5 respectively, led each time within 24 hr. to the almost complete destruction of the skin reactive components of the pollen extracts; and, contrary to reports by *Piper* (10), pepsin digestion significantly reduced the clinical efficacy of pollen extracts (9). The carbohydrate and pigment moieties on the other hand, appear to be unnecessary for both skin reactivity and clinical efficacy; colourless protein fractions of very low carbohydrate content have been isolated by acid salt fractionations (1) and were, weight for weight, at least as active, cutaneously and clinically, as the strongly pigmented carbohydrate rich components (8).

Some degree of fractionation between skin reactivity and one rabbit antibody precipitating pollen antigen could be achieved by prolonged electrophoresis, but there was evidence of a breakdown into smaller molecules (3). Similarly, heating of pollen extracts at 100° destroys their ability to form precipitates with rabbit antisera; within 1 min. at 100° an inactive precipitate forms while the filtrate proved clinically as active as the unheated solution (9); skin reactivity only gradually breaks down with [illegible]

[illegible] unadulterated pollen extracts with these same antisera. These breakdown products could be concentrated by ultrafiltration, i.e. they are presumably of a molecular weight above 10,000; they have therefore been called "incomplete antigens"

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rather than haptens (5). It is possible that such "incomplete antigens" are clinically active.

The evidence given so far is compatible with the view that skin reactive pollen components are also clinically active. From previous work (3) it would appear that reagins and rabbit antibodies may be formed in response to different parts of one and the same antigen. This idea is particularly attractive in connection with the theory that allergenic materials are substances small enough to act as haptens, yet big enough to act on other occasions as full antigens. As haptens, i e. combined with a carrier in a possibly uncoiled condition, such molecules may well present a different part of their structure as a matrix for antibody formation from the uncombined molecule acting as an antigen in its own right (2).

However, these are no more than working hypotheses. The possibility remains that different molecules (and not different parts of the same molecule) are responsible for the formation of rabbit antibodies on the one hand, and reagins in man on the other hand, or that only some of the substances antigenic in rabbits are allergenic in man.

From antigen-antibody precipitation studies in gel (Ouchterlony tests) we know that all the British grass pollens so far examined by us (*Festuca rubra*, *F. pratensis*, *F arundinacea*, *Avena arrhenatherum*, *Lolium multiflorum*, *Anthoxanthum odoratum*, *Phleum pratense*, *Alopecurus pratensis*, *Dactylis glomerata*) have a number of antigens (for rabbits) in common and that all of them contain a common heat labile antigen. The latter appears to be the main antigen, for it is always the first to produce antibodies in rabbits This heat labile antigen has therefore been called by us antigen "A".

So far it has not been possible to isolate antigen A. Nevertheless we have felt justified in assaying pollen extracts in terms of this easily identifiable antigen which is closely associated with skin reactivity, even if it should eventually prove not to be the main allergen, or, indeed, not an allergen at all Assays in terms of a common antigen closely associated with skin reactivity are certainly preferable to assays in terms of purely chemical analyses, such as total nitrogen or phosphotungstic acid precipitable nitrogen, quantities which are not clo[illegible]
antigen A appears to
and is therefore a s
reactions in general

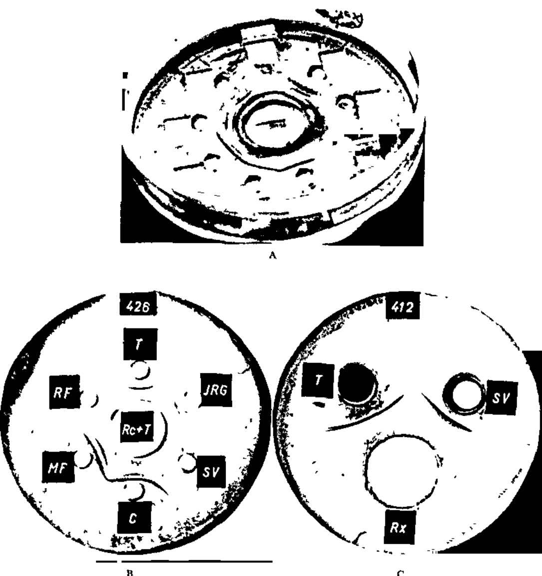


Fig. 1. Ouchterlony tests on grass pollen extracts. The big middle compartments contain rabbit antisera, R_T against Timothy *(Phleum pratense)*, R_{GT} - R_X, against Timothy + Cocksfoot *(Dactylis glomerata)*. The peripheral cups contain pollen extracts. RF = Red fescue *(Festuca rubra)*, TOG = Tall oat grass *(Arrhenatherum elatius)*, SV - Sweet vernal *(Anthoxanthum odoratum)*, M Fox = Meadow foxtail *(Alopecurus pratensis)*, T = Timothy, C = Cocksfoot, IRG = Italian ryegrass *(Lolium multiflorum)*, TF Tall fescue *(Festuca arundinacea)*, MF = Meadow fescue *(Festuca pratensis)*. Numerous common antigens, but some peculiar to only one of the grasses (e.g. for T, bundle of unshared lines near the antibody cup in A). Although it would seem from A and B that SV does not share the common main antigen, it is clear from C that SV merely contains a smaller amount of it; the same was found to be true for RF when quantitative tests were carried out.

Augustin, R. Int Arch Allergy 11 223 (1957)

Demonstration, by Gel-Diffusion, of an Instance of Drug Allergy

By R. Augustin

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Skin tests are useless in patients with drug allergies. This is not surprising, considering (1) the haptenic nature of drugs (2), that probably cellular antibodies only are formed, and (3) that humoral antibodies, if produced at all, may have been formed to a drug-body protein conjugate (*R. Augustin*, 1955). Apart from *R Hoigné*'s light scattering method (1956) which is still under test, an objective test method is thus lacking. The following experimental results seem therefore worth reporting.

Through the courtesy of *Dr. Mowbray* (St. Mary's Hospital) serum from a patient allergic to phenolphthaleine was obtained. The fresh serum was said to form a precipitate with phenolphthaleine. Although this could not be repeated a fortnight later, a somewhat diffuse but clearly marked precipitation line was formed in agar when phenolphthaleine mixed and incubated with normal human serum, was allowed to diffuse against the patient's serum. Phenolphthaleine itself, in a large range of different concentrations, did not give a visible reaction. Attempts to imitate this reaction with pollen extracts and reagins, and other drugs *versus* the serum of sensitive patients, have so far failed.

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Although cutaneous tests and clinical trials must remain the basis for all work on standardisation, they are too inaccurate for quantitative work. Also, standardisation in terms of any one well defined component is preferable to standardisation by skin tests alone. Skin tests record the sum of an unknown number of skin reactive components; the number and amounts of these activities must necessarily vary with the sensitivity spectrum of the (allergic) subject used, as well as with the composition (in terms of active components) of the particular pollen employed as a standard. Variable results must thus be expected, quite apart from uncontrolled variations in size of response due to variations in temperature, site of reaction etc.

Our ultimate aim must naturally be to have a standardisable antibody (or antigen) which is directly related to skin reactivity and clinical sensitivity.

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Demonstration, by Gel-Diffusion, of an Instance of Drug Allergy

By R. Augustin

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Skin tests are useless in patients with drug allergies. This is not surprising, considering (1) the haptenic nature of drugs (2), that probably cellular antibodies only are formed, and (3) that humoral antibodies, if produced at all, may have been formed to a drug-body protein conjugate (*R. Augustin*, 1955). Apart from *R. Hoigné*'s light scattering method (1956) which is still under test, an objective test method is thus lacking. The following experimental results seem therefore worth reporting.

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Although cutaneous tests and clinical trials must remain the basis for all work on standardisation, they are too inaccurate for quantitative work. Also, standardisation in terms of any one well defined component is preferable to standardisation by skin tests alone. Skin tests record the sum of an unknown number of skin reactive components; the number and amounts of these activities must necessarily vary with the sensitivity spectrum of the (allergic) subject used, as well as with the composition (in terms of active components) of the particular pollen employed as a standard. Variable results must thus be expected, quite apart from uncontrolled variations in size of response due to variations in temperature, site of reaction etc.

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nal pathology, since new cases were observed all over the world among oil-mill workers, fertilizer retailers, dockers, draymen, farmers and people in contact with bags which had simply been filled with castor beans (*Lucchese, Ordman, Kaufmann, Bernton, Steiner, Figley* and *Rawling, Small*).

Lastly, epidemics of asthma had been mainly described among citizens in towns where castor oil-mills were in existence, e.g. Toledo, Ohio, USA (*Figley*), Bauru, Brazil (*Mendes* and *Ulhoa-Cintra*), Johannesburg, South Africa (*Ordman*). Similar cases were also observed in Italy (*Berto* and *Bassi*) and in Germany (*Steiner, Hansen*).

A Material and Allergological Investigations

1. All our patients were genuine allergic people, mostly asthmatics (83 cases), some of them suffering only from allergic rhinitis (15 cases) or spasmodic cough (4 cases).

The material consists of 81 adults (37 women and 44 men), 21 children (8 girls and 13 boys).

All these patients systematically tested with extracts of

1. Ordinary food stuffs (eggs, milk, chocolate, potatoes, wheat). Sensitization was observed only in a very small number of cases.

2. Ordinary local pollens which enabled us to trace one case of allergy to parietary, 4 cases of allergy to planetree pollen and 25 cases of allergy to grasses. We only found one case of sensitization to Ricinus communis pollen in a patient who grew castor in her garden.

3. Moulds, Hollister's 33 mould types. In this group, there were positive tests to various moulds (61 cases out of 88).

4. Bedding material (feathers, wool, kapok). Allergy to feathers was sometimes observed.

5. *House dust (Hollister-Stier extract). This was the most frequently observed allergy, since all our patients, except a few (14), were sensitive to dust extract in various degrees* (+ to ++++).

6. Various seeds. Allergy to pyrethrum or mustard seeds is rare, but *cottonseed allergy was sometimes observed (16 cases), as well as flaxseed allergy which was more frequent (41 cases). Castor bean tests were of course strongly positive in all cases* (++ to ++++).

Panzani, R Int Arch Allergy *11* 224—236 (1957)

Respiratory Castor Bean Dust Allergy in the South of France with Special Reference to Marseilles

By RAPHAEL PANZANI, Marseilles

Since 1910, due to the work of *Schern*, it has been known that castor bean allergy occurs in animals. Since 1914 due to the auto-observation by *Ahlaire* who ascribed his asthma to Ricin allergy it has been known to occur in man. The first case in Marseilles was described in 1951 in an address given together with Prof. *Olivier* to the «Société Provençale de Médecine de Travail». We thought first that it was mostly among workers in contact with castor bean and its derived products (dockers, oil-mill workers) that asthma could be observed, but we had to change our hypothesis very rapidly, as can be inferred from the following history. In *1951*, a 35-year-old patient suffering from light allergic rhinitis came to live in Marseilles at «Le Corbusier», a building in the immediate vicinity of an oil-mill. Asthma was observed within a month and the patient rapidly became inured to various therapeutics and to hyposensitization to house dust, to which he was allergic. Other tests had been practised with moulds, flaxseed, various textile products, pyrethrum, but they were all negative. Thinking of a possible castor bean allergy, we made a scratch-test with this allergen, causing a strong local reaction and even a fit of asthma. The patient left Marseilles and since then has been cured of his asthma. From that time onwards our experiments have been enlarged and we will present *102* cases of castor bean respiratory allergy.

History

Castor bean respiratory allergy was first described among laboratory people who had to handle castor beans for a long time (*Ahlaire, Snell, Bernton, Robins, Woringer*). It then touched professio-

3. *Castor Oil Extraction*

In France, this extraction is only practised in four oil-mills scattered round Marseilles and until 1937 it was extracted by a mechanical process which yielded about 6 to 8% oil in the cakes. The cakes were therefore wet and non-powdery. Nowadays, a chemical process is used which yields a much drier and more powdery cake but which is also much more dangerous.

4. *Intoxication by Castor Oil Cakes*

Castor oil is absolutely non-poisonous but the oil cakes containing ricin are dangerous for those who handle them. All those working in that industry are well acquainted with the symptoms of acute intoxication by ricin (commonly called "Ricin disease") which resembles common flu with respiratory catarrh, mainly oto-rhino-laryngitis, fever, pains, asthenia and sometimes urticaria. This complaint, which is easily cured, only appears when the subject is exposed to thick dust.

The allergical phenomena caused by castor oil cake dust are well known to the Marseilles mill-owners who eliminate possible asthmatic workers. Tests made on workers and employees in a certain oil-mill in Marseilles have always proved negative.

5. *The Use of Castor Oil Cakes in the Country*

These oil cakes are first-rate fertilizers and are only used in the South of France (Comtat Venaissin, Bouches du Rhône, district of Hyères, Antibes) which allows the production of two or three crops of vegetables every year. As this fertilizer needs both heat and water for its action, it cannot be used in other French provinces for market-gardening (Britany). Oil cakes are used either in their natural state or in association with other fertilizers, or with straw and ammonia in order to obtain a synthetic fertilizer. The latter procedure will probably prevail in the future.

These oil cakes are scattered on the ground at certain times of the year (spring and the end of summer), thus respiratory castor bean allergy is apt to assume a recurrent character simulating pollinosis or mould allergy.

6. *Castor Beans in France and Elsewhere*

After they have been gathered the castor beans are sent in sacks by boat to Marseilles where they are discharged and taken

In five cases (including two children) severe systemic reactions (asthma, urticaria or incipient shock) appeared after the test. We always used the scratch method and never made puncture or intradermal reactions, which are more dangerous.

Commercial extracts available from laboratories, are not absolutely free from ricin, which is a very strong poison, so that during the next ten days after the test a toxic skin reaction was produced. It was pseudo-inflammatory, had nothing to do with castor-bean allergen but was due to ricin. We found an obvious proof of this fact by submitting ten of the non-allergic subjects to a Prausnitz-Kustner test which was absolutely negative.

None of our patients ever suffered from the absorption of therapeutic castor oil. To our knowledge, only one case of this type of allergy has been described by *Blank*.

B. The Castor Bean Industry and Etiology of Our Observed Cases

1. Castor

Castor or "Ricinus communis" belongs to the Euphorbiaceae. It is often a rather tall shrub and is essentially grown in sub-tropical countries where it sometimes appears spontaneously (more especially in South Africa and Brazil which is the first world producer, secondarily in Siam, Haiti, Central America). Attempts to cultivate castor have been made in the States, Northern Italy and the South West of France (Castelsarrasin). In the South of France, it is found in the gardens and also has a useful purpose as it is reputed to drive away mosquitoes. Castor gets pollen from June to October. Its pollen, as we have seen, only rarely produces allergy. We were mostly interested in the castor fruit and its bean-shaped almond.

2. Expansion of the Castor Bean Industry

Castor beans yield an oil which has growing industrial uses, e. g. paint, ink, cosmetics, explosives, and mostly plastic products, among which "Rilsan" of the Organico Society is the most recent product. In the same way, the need for organic animal manure is felt in the country of our province and invites the farmers to make greater use of castor oil cakes, an excellent fertilizer, especially for market-gardening.

bronchial catarrh which usually appears or disappears unexpectedly, and in most cases without expectoration. *This type of asthma is made worse by the most frequent wind in our town called "Mistral"* but it improves when it rains. It will sometimes completely disappear on Sundays when the oil-mills are closed and specially when the patient leaves the town. It is possible to assert that asthma only existing in Marseilles is very probably due to castor-beans but in the country it worsens during spring and summer when the oil-cakes are scattered in the fields. However, it improves when the patient leaves his home.

In 25% of these cases a fit of asthma is accompanied by urticaria (sometimes generalised urticaria) which gives castor-bean allergy a similar appearance to parietary pollen allergy.

2. Castor Bean Scratch Tests

The diagnosis rested on strongly positive scratch-tests. Severe systemic reactions, as we have already stated, appeared sometimes immediately, while local pseudo-inflammatory reactions which were due to ricin constantly appeared at later periods.

3. Local Passive Transfer of Antibodies

The Prausnitz-Kustner reactions were practised in 12 cases (8 adults and 4 children) according to the usual technique. The castor bean test was made by the puncture method advocated by *Small*. The results were constantly positive (+++ or ++++) and never entailed constitutional reactions. Local pruritus was interesting to observe, and often lasted 8 days after the reaction.

4. Experimental Asthma

In 14 patients (10 of whom had only distant contact with castor beans) we were able to cause manifestations of respiratory allergy.

a) 10 patients inhaled very finely crushed castor-oil cake dust projected by a blast engine;

b) 4 patients inhaled ricin allergen from the Hollister laboratories (also used in scratch-tests), diluted in 100 times its own volume of physiological saline ($^1/_{1000}$).

to the Marseilles oil-mills. After extraction, the oil cakes which are still in sacks are sent by car or train to market-gardening districts. The contaminated sacks sometimes are used to carry potatoes or coffee, whence new danger of contamination arises. Different workmen will, in that way, be exposed to this allergy e. g. sailors, dockers, draymen, fertilizer retailers, people dealing with these sacks and mending them, especially farmers and oil-mill workers. Farmers are very much exposed to contamination on account of the frequent winds in our country which carry the dust about, as are the inhabitants of Marseilles who live in the near vicinity of the oil-mills. It should also be remembered that France imports castor oil cakes from Holland and Germany and railway workers are consequently exposed.

In our study, we have been able to trace the following etiological cases:

1. Laboratory people, working on castor beans or castor oil cakes:	1 case
2. Dockers, workers on the wharves, and drivers:	5 cases
3. People growing and gathering castor beans:	3 cases
4. Workers and oil-mill employees:	1 case
5. Fertilizer retailers, bag merchants, people living in their vicinity:	7 cases
6. Country people handling castor oil cakes, and inhabitants of the country:	20 cases
7. Citizens of Marseilles living in the vicinity of oil-mills where the dust has been scattered by the wind	65 cases

It should also be noted here that about 10 of those patients live within 2 or 6 kilometers of the oil-mills. We wondered whether the positive scratch-tests in these cases were a manifestation of a pre-clinical humoral allergic condition and not a present clinical condition. We noticed that this was not the case.

C. Clinical and Allergological Diagnosis of Respiratory Allergy to Castor Beans

1. Clinical Characteristics

After a clinical examination had yielded no sign of castor bean sensitization, an interview brought us interesting information. *We generally dealt with asthma of the pollinical type,* i.e. an oculo-nasal-

cakes). In the latter clinical form, which is the most frequent in our study (70 cases), castor bean allergy is only one minor element.

The importance of castor bean allergy in a population of asthmatics is comparatively small. About 100 cases out of 1050 asthmatic, allergic patients tested are likely to show a sensitivity to castor beans for various reasons. The opposite test which was always negative has been performed in 200 allergic asthmatics living in French provinces where castor beans are unknown.

This seems to show that castor bean dust is not a powerful sensibilizer in man under normal clinical conditions, but as *Ratner* has shown, it is in animals.

Castor bean allergen is consequently quite distinct from ricin though it is impossible to obtain absolutely ricin-free allergenic extracts for diagnosis and treatment. The allergen is to be found in the bean and bark of leaves and flowers It is thermostable, can be dissolved in water, precipitated into alcohol and dialysable by ultra-centrifugation – characteristics which differentiate it from ricin.

Coulson, Spies and *Stevens* have isolated the C. B. 1A from ricin which possesses the antigenic properties of castor oil cakes in men and animals They have also isolated C. B. 65A which is twelve times less active than C. B. IA in sensitizing guinea-pigs experimentally. The same writers add that "Chemical properties and amino-acid content of the castor bean allergen were remarkably similar to those of the previously described cotton-seed allergen. Both were characterised by a relatively high proportion of arginine and cystine. However, C. B. IA contained no tryptophane, in contrast to the cotton-seed allergen which contained 1.4% of this amino-acid."

D. *Treatment*

Apart from antihistamines which are not always efficient, corticoid hormones of transitory action and non-specific desensitising treatments which can only bring about slight improvements, the treatment of respiratory castor bean allergy rests on the following triad:

a) Suppression of the allergen;
b) Specific hyposensitization to associated allergens;
c) Specific hyposensitization to the castor bean.

In the first case, the castor bean oil cakes were pulverised for an hour in a small room before the patient's arrival. The patient had not been warned beforehand and had shown no signs of asthma for a fortnight. Five to twenty minutes after entering *the room, he had a fit of asthma starting with oculo-nasal catarrh.* Immediately after leaving this environment the patient felt his asthma progressively disappear within one or two hours. Only in three cases was it necessary to inject theophylline together with an antihistamine in order to soothe the dyspnea.

In the second case, the experiment was still more conclusive, and especially more rapid. 3 to 15 minutes after inhalation of an aerosol which lasted from 20 seconds to one minute, a violent fit of asthma developed which could only be soothed by anodynes. It was impossible, of course, in those cases to eliminate a psychic factor.

At the same time, 6 young adults suffering from allergic asthma came to visit us imagining they suffered from castor bean allergy. They showed no immediate reaction after a ricin-allergen aerosol given for three minutes. The next day and for two or three days afterwards catarrh developed in the upper respiratory tracts simulating the castor-bean workers' flu and very probably due to ricin.

All those allergological and experimental investigations lead to the assertion that *a positive scratch-test to castor beans in an asthmatic patient is sufficient proof of his suffering from a clinical allergy to that bean.*

The frequency of the associated allergies we have described above enables us to conclude that the castor bean factor is essentially variable according to the patient. We are thus able to isolate three clinical forms:

1. *Isolated allergy to castor beans* appearing in subjects very much exposed either in their profession or at home presents the most dangerous and the less frequent case, unless the patient can change either his profession or his home: 14 cases.

2. *Castor bean allergy associated with house dust and mould allergy appearing in over-exposed subjects* (country people, dockers, fertilizer retailers or immediate neighbours of oil mills): 18 cases.

3. *Castor bean allergy associated with moulds or house-dust allergy, in less exposed subjects* (citizens of Marseilles or of the surrounding country remote from sources of dust, castor beans or castor oil

in that way improved with more ease and speed than the other test-patients It is, of course, too early to judge methods which are apt to imply an important psychotherapeutic effect.

In conclusion, castor bean dust respiratory allergy is a danger to asthmatics living in the South-East of France and in Marseilles, but it is a rather rare eventuality since it has only been found in about 10% of our asthmatics. It has been said that "Ricinallergen" could easily be sensitizing. This seems to be an exaggeration since a great number of our asthmatic patients were not sensitized although they were under the same unfavourable circumstances.

Castor bean allergy is only one element of the more complex allergies in which house-dust, moulds, and to a lesser degree flax-seed and cottonseed, play a part. It is easily diagnosed. The castor bean factor, however, is much more difficult to appreciate. Except in extreme cases where the castor bean allergy is isolated it is necessary to wait for the results of dust and mould hyposensitization to decide. An ideal solution would of course be to take the patient away from contact with castor bean dust, or to make it thinner and less aggressive. Specific castor bean hyposensitization according to the usual technique, is still too dangerous to be commonly applied. At the present moment, we use homoeopathic doses of "Ricinus communis" per os, until the technique of hyposensitization by means of castor bean allergen aerosols will be perfected.

Summary

The danger for asthmatic people discussed:

1. of the castor-oil industry in Marseilles, as the oil-mills are situated in the residential districts of the town;

2. of the general use in the South-East of France of castor bean oil cakes as a fertilizer for market-gardening. The author stresses the fact that since 1951, when his first medical observation on this subject was published, he has been able to collect about a hundred cases of respiratory castor bean dust allergy.

This disease is often observed among persons in direct contact with castor beans or oil cakes (country people, dockers [illegible] retailers, oil-mill [illegible]

Marseilles

I. It is difficult to suppress the allergen. However, the following measures can be apllied:

1. Use of wet oil cakes retaining their oil. This measure, however, would have to be applied on an international plane because 6% to 8% of oil retained in the oil cakes represents part of the benefits to the oil-mill owners.

2. Use of powerful aspirators in the oil-mills at the same time taking care not to scatter dust about. This is done in California by the Baker Oil Co.

3. Humidification of the oil cakes to 12%. They cannot be humidified to a higher percentage on account of fermentation.

4. Use of paper bags which are water-proof as the usual cloth bags are too porous and allow the finest dust to sift through.

5. To avoid storage of oil cakes in farms, and taking care that the wholesale dealers are situated far from residential districts.

6. Building oil-mills far from big towns and using other fertilizers in the country are too idealistic measures for practical application.

7. Air conditioners used in houses situated near the oil mills allow comparative quiet for the patient while at home but on leaving he is again exposed to the allergen.

II. Specific hyposensitization to associated allergens is easily realised since these are mostly house dust and moulds. This usually improves the condition considerably, the "Castor bean" factor being the less important. It may be sufficient to cure a few of the less exposed patients, but it proves absolutely inefficient in more severe cases.

III. Specific hyposensitization to castor beans is not generally advisable because commercial extracts are not absolutely ricin-free, as has already been stated. In spite of this some authors have attempted to use the common method of intradermal injection of progressive doses, either under protection of cortisone or not. A discrepancy has, however, appeared in the results.

Herxheimer reported his observation of a desensitized patient by employing the bronchial method which consists in having the allergen inhaled in aerosols under spirometric control. We thought this method reasonable and proceeded with a few experiments which "a priori" seemed comparatively satisfying. Meanwhile, we thought of using homoeopathic doses of "Ricinus communis" at the 4th centesimal, letting the patient take two or three granules of this dilution each morning and evening. The 30 patients treated

et obtenu par un aérosol d'une solution de ricinallergène une crise d'asthme expérimentale.

L'hyposensibilisation spécifique par voie générale qui a été tentée, nous paraît devoir être, pour l'instant, rejetée à cause de ses dangers et de ses incertitudes, mais nous nous proposons de faire l'hyposensibilisation par voie bronchique suivant la méthode du docteur *Herxheimer* en utilisant, par aérosols, des doses croissantes d'allergène. En général, l'éloignement des sources de poussières de ricin et l'hyposensibilisation aux poussières de maison et aux moisissures suffisent à améliorer les malades.

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bean allergy (i.e. asthma, allergic rhinitis or more generally their associated symptoms) is sometimes observed as an isolated phenomenon, but in most cases it is combined with house dust, mould, flaxseed or cottonseed allergy. Scratch tests were always strongly positive (+++ or ++++) and sometimes caused severe systemic reactions. *Prausnitz-Küstner* reaction had in some cases been practised with obviously positive results while castor-allergen aerosols never failed to produce experimental fits of asthma. Castor pollen tests were in only one case positive. General specific hyposensitization which has been attempted should at the present moment be discarded on account of its dangers and technical insecurity. We hope to practise bronchial hyposensitization according to *Herxheimer*'s method with increasing doses of allergen in aerosols. Removal of the patients to a reasonable distance from the sources of castor bean dust, and hyposensitization to house dust and moulds are usually enough to bring about substantial improvement.

Résumé

L'auteur insiste sur les dangers que représentent pour les asthmatiques, d'une part l'industrie du ricin à Marseille, où les huileries sont situées dans la ville même et, d'autre part, l'utilisation dans le Sud-Est de la France des tourteaux de cette graine comme engrais des terres destinées à la culture maraîchère. En effet, depuis 1951, date à laquelle l'auteur a publié la première observation marseillaise, une centaine de cas d'allergie respiratoire aux poussières de ricin, a pû être réunie.

Cette affection se rencontre chez des sujets qui ont un contact direct avec les graines où les tourteaux de ricin (paysans, dockers, marchands d'engrais, ouvriers des huileries etc.) et même chez les habitants de Marseille voisins plus ou moins immédiats des huileries. L'allergie respiratoire au ricin (asthme, coryza spasmodique ou, généralement les deux associés) est quelquefois isolée; elle s'associe, le plus souvent, à une allergie aux poussières de maison, aux moisissures, à la graine de coton et à la graine de lin. Les tests allergiques en cuti-réaction sont toujours fortement positifs (+++ ou ++++) et donnent quelquefois des réactions générales impressionnantes. Nous avons pratiqué, dans quelques cas, une réaction de *Prausnitz-Kustner* qui a été franchement positive

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